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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT) -

(51) International Patent Classification 6 : C12N 15/12, C07K 14/47

A2

(11) International Publication Number: WO 99/28465

(43) International Publication Date: 10 June 1999 (10.06.99)

(21) International Application Number: PCT/US98/25316

(22) International Filing Date: 27 November 1998 (27.11.98)

(30) Priority Data:

60/069,251	28 November 1997 (28.11.97)	US
60/095,950	10 August 1998 (10.08.98)	US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PIAS MOLECULES THAT RECOGNIZE AND BIND STAT PROTEINS AND USES THEREOF

(57) Abstract

The invention provides a family of proteins named PIAS which function as specific inhibitors of STAT proteins. Methods of using PIAS molecules are also provided to regulate STAT proteins.

[redacted]

ATTORNEY DOCKET NUMBER:10873-0008
SERIAL NUMBER: 09/492,764
REFERENCE: AI

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PIAS MOLECULES THAT RECOGNIZE AND BIND STAT PROTEINS AND USES THEREOF

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to describe more fully the state of the art to which this invention pertains.

FIELD OF THE INVENTION

The invention is directed to novel protein inhibitors of activated STAT (PIAS) and methods for making and using such proteins.

BACKGROUND OF THE INVENTION

Activation of early response genes by interferons (IFNs) and other cytokines requires tyrosine phosphorylation of a family of transcription factors termed signal transducer and activator of transcription (STAT) proteins. STAT proteins relay signals from activated cell surface receptors directly to the nucleus and have been demonstrated to play a critical role in gene induction by a variety of hemopoietic cytokines and hormones.

The STAT protein is activated by the gp130 family of cytokines, e.g., the interleukin 6 (IL6) family of cytokines, epidermal growth factor, and leptin. Tyrosine-phosphorylated STAT3 binds to a specific DNA sequence in its target genes (Zhong et al. (1994); Akira et al. (1994)) and participates in signal transduction pathways activated by the IL6 family of cytokines and by epidermal growth factor (Zhong et al. (1995); Akira et al. (1994)). STAT3 is also activated in cells treated with leptin, a growth hormone that functions in regulating food intake and energy expenditure (Zhang et al. (1994)). Targeted disruption of the mouse gene encoding STAT3 leads to early embryonic lethality (Takeda et al. (1997)). Like other members of the STAT family, STAT3 becomes tyrosine phosphorylated by Janus kinases (JAKs). Phosphorylated STAT3 then forms a dimer and translocates into the nucleus to activate specific genes (Darnell et al. (1994)).

The invention relates to a family of protein inhibitors of activated STAT (PIAS) molecules that directly inhibit STAT function. This family includes but is not limited to, PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy. Sequence analysis indicates that human PIAS1 is almost identical to a previously reported human protein named GBP (Gu/RH-II binding protein) (Shuai et al. Nature (1993)). However, GBP lacks 9 amino acid residues when compared with PIAS1. Further, GBP does not function as an inhibitor of STAT but was identified as a putative interaction protein of Gu/RNA helicase II.

SUMMARY OF THE INVENTION

This invention includes novel PIAS molecules that are STAT binding molecules (e.g., STAT1 and STAT3). The binding of PIAS molecules to STAT indicates that STAT signaling pathways can be suppressed at multiple steps, in a general or specific manner. It seems that the overall strength of STAT signaling for a given cell type may be largely affected by the relative level of STAT protein and PIAS expression. Therefore, these molecules can be used for the detection and treatment of diseases associated with STAT mediated cellular responses.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1a and 1b provides the primary sequence and expression of PIAS3and a photograph of a gel (northern blot) showing expression of PIAS3 mRNA in human tissues, respectively.

Figures 2a and 2b are photographs of protein immunoblots showing the in vivo interaction of PIAS3 with STAT3.

Figures 3a and 3b are photographs of electrophoretic mobility shift assays showing the inhibition of the DNA-binding activity of STAT3 by PIAS3.

Figures 4a-4c are bar graphs showing the effect of PIAS3 on STAT-mediated gene activation.

Figure 5 is a sequence comparison of the PIAS family of proteins including mPIAS3, hPIAS3, hPIAS1, mPIAS1, hPIASxα, hPIASXβ, and hPIASy.

Figures 6a and 6b are bar graphs and photograph showing that PIAS1 inhibits STAT1-mediated gene activation.

Figure 7 is a photograph showing that PIAS1 inhibits the DNA binding activity of STAT1.

Figures 8a-e are photographs showing the specificity of PIAS-STAT interaction.

Figures 9a-b are photographs showing phosphorylation on Tyr-701 of STAT1 is required for PIAS1-STAT1 interaction.

Figure 10 is the amino acid and nucleic acid sequences of murine PIAS1 having a mutation at amino acid position 374 (Cys→Ser).

Figure 11 is the amino acid and nucleic acid sequences of murine PIAS3 having a mutation at amino acid position 349 (Cys→Ser).

DETAILED DESCRIPTION OF THE INVENTION

PROTEINS OF THE INVENTION

The invention relates to the PIAS family of proteins. PIAS molecules bind STAT proteins. Typically, the STAT so bound is phosphorylated and the phosphorylation (e.g., tyrosine phosphorylation) is cytokine induced.

In some embodiments, a PIAS molecule will specifically bind a member of the STAT family of proteins but not another. For example, the invention provides isolated PIAS

molecules that bind and inhibit STAT1 but do not bind STAT3. By binding STAT1, these proteins block the DNA binding activity of STAT1 thereby resulting in the inhibition of STAT1 mediated gene activation. In another example, the invention provides isolated PIAS molecules that bind and inhibit STAT3 but do not bind STAT1. By binding STAT3, these proteins block the DNA binding activity of STAT3 thereby resulting in the inhibition of STAT3 mediated gene activation.

PIAS molecules described herein include PIAS from any species, e.g., mammalian, including bovine, ovine, porcine, murine, equine and human. PIAS molecules may be in a naturally occurring form or from any source whether natural, synthetic, semi-synthetic or recombinant. PIAS molecules can be embodied in many forms, preferably, in a purified or isolated form. PIAS molecules include those embodiments having the sequences disclosed herein including homologues, isoforms, allelic variants and conservative substitution mutants thereof that have STAT binding activity and can be isolated/generated without undue experimentation following the methods outlined below. For the sake of convenience, all PIAS molecules will be collectively referred to as the PIAS molecules, the proteins of the invention, or PIAS.

Particular embodiments of the PIAS family of proteins include but are not limited to PIAS1 and PIAS3.

PIAS1 binds and inhibits STAT1 but does not bind STAT3. By binding STAT1, PIAS1 blocks the DNA-binding activity of STAT1 thereby resulting in the inhibition of STAT1 mediated gene activation. STAT1 can be in the form of a homodimer or a heterodimer. Additionally, STAT1 can be STAT1(alpha) or STAT1-ß. Human PIAS1 (hPIAS1) has the sequence shown in Figure 5. Murine PIAS1 (mPIAS1) has the sequence shown in Figure 5. Included within the scope of the present invention are alleles (or allelic variants) of PIAS1. As used herein, an allele is an alternative form of PIAS1. Alleles result from a mutation, i.e., a change in the nucleic acid sequence and generally produce altered mRNAs or proteins whose structure of function may or may not be altered.

PIAS3 binds and inhibits STAT3 but does not recognize and bind STAT1. By binding STAT3, these proteins block the DNA-binding activity of STAT3 and STAT3 mediated cellular responses, e.g., STAT3 and IL6 mediated cellular responses. Blocking the DNA-binding activity of STAT3 results in the inhibition of a STAT3 transactivating/signaling complex. STAT3 can be in the form of a homodimer or heterodimer. Additionally, the STAT3 can be STAT3(alpha) or STAT3-ß.

One embodiment of PIAS3 proteins is murine PIAS3 (mPIAS3) having the sequence shown in Figure 5. Another embodiment of PIAS3 proteins is human PIAS3 (hPIAS3) having the sequence shown in Figure 5. Included within the scope of the present invention are alleles of PIAS3.

Another embodiment the PIAS molecule is PIASxα. Human PIASxα (hPIASxα) has the sequence shown in Figure 5.

A further embodiment of the PIAS molecules is PIASxβ. Human PIASxβ (hPIASxβ) has the sequence shown in Figure 5.

Also, an additional embodiment of the PIAS molecules is PIASy. Human PIASy (hPIASy) has the sequence shown in Figure 5.

In addition to allelic variants of PIAS molecules, the invention also encompasses conservative substitution mutants of PIAS molecules. For example, changes to the primary amino acid sequence of the PIAS molecules are possible so long as the resulting protein maintains the ability to function as a specific inhibitor of STAT protein (e.g., Figures 10 and 11). Changes include amino acid substitutions. Amino acid substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative". For example, it is a well-established principle of protein chemistry that certain amino acid substitutions, entitled "conservative amino acid substitutions," can

frequently be made in a protein without altering either the conformation or the function of the protein. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa.

Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments and are encompassed by the invention.

The PIAS family of proteins, e.g., the PIAS1 and PIAS3 proteins discussed herein, exhibit significant homology (over 50%) with each other. Even more significant homology (over 70%) is found when comparing the area n-terminus to the putative zinc binding motif between the PIAS1 and PIAS3 members.

PIAS molecules have several highly conserved domains, including a putative zinc binding motif present and a highly acidic region (Fig. 5). The COOH-terminal regions of PIAS molecules are the least conserved. For example, PIASxα and PIASxβ are identical, except in their COOH terminal regions.

The invention further provides portions of the PIAS molecules of the present invention. As used herein, a portion of a PIAS molecule refers to a small portion of the entire PIAS sequence. Preferably, it is a portion or the entire area of the N-terminal domain of PIAS, i.e., the portion of PIAS that can inhibit STAT. Typically, it is the area located N-terminal

to the zinc binding motif of PIAS. A portion of the N-terminal domain of PIAS includes, but is not limited to, amino acid positions 1 to 425 of PIAS1 and PIAS3; amino acid positions 1-133 of PIAS1 and PIAS3; and amino acid positions 50-168 of PIAS1 and PIAS3). The size of the portion will be determined by its intended use.

For example, if the portion is to be used as an immunogen, then a fragment length is chosen so as to generate antibodies directed against that portion of PIAS. Portions of PIAS that are particularly useful can be readily identified from the entire PIAS sequence using art-known methods.

PIAS ANTIBODIES

The invention further provides antibodies that bind to PIAS. The most preferred antibodies will selectively bind to PIAS and will not bind (or will bind weakly) to non-PIAS. Anti-PIAS antibodies that are particularly contemplated include monoclonal and polyclonal antibodies as well as fragments containing the antigen binding domain and/or one or more complement determining regions of these antibodies.

In one embodiment, the PIAS antibodies specifically bind to the area n-terminal to the zinc binding motif of PIAS. In other embodiments, the PIAS antibodies specifically bind to other domains of PIAS. As will be understood by those skilled in the art, the regions or epitopes of PIAS to which an antibody is directed may vary with the intended application.

The invention also encompasses antibody fragments which specifically recognize PIAS. As used herein, an antibody fragment is defined as at least a portion of the variable region of the immunoglobulin molecule which binds to its target, i.e., the antigen binding region. Some of the constant region of the immunoglobulin may be included.

PIAS antibodies may also be used in methods for purifying PIAS and peptides and for isolating PIAS homologues and related molecules. For example, in one embodiment, the method of purifying PIAS comprises incubating a PIAS antibody, which has been coupled to a solid matrix, with a lysate or other solution containing PIAS under conditions which permit the PIAS antibody to bind to PIAS; washing the solid matrix to eliminate impurities; and eluting the PIAS from the coupled antibody.

Other uses of the PIAS antibodies of the invention include generating anti-idiotypic antibodies that mimic PIAS.

Various methods for the preparation of antibodies are well known in the art. For example, antibodies may be prepared by immunizing a suitable mammalian host using PIAS, peptide, or fragment, in isolated or immunoconjugated form (Harlow, Antibodies, Cold Spring Harbor Press, NY (1989)). In addition, fusion proteins of PIAS may also be made and used, such as a PIAS GST-fusion protein. One embodiment of a PIAS fusion protein would be a N-terminal PIAS protein (e.g., from amino acid positions 1 to 425 or 1 to 133 (Figure 5)). Cells expressing or overexpressing PIAS may also be used for immunizations. Similarly, any cell engineered to express PIAS may be used. This strategy may result in the production of monoclonal antibodies with enhanced capacities for recognizing endogenous PIAS.

The amino acid sequence of PIAS presented herein may be used to select specific regions of PIAS for generating antibodies. For example, hydrophobicity and hydrophilicity analyses of the PIAS amino acid sequence may be used to identify hydrophilic regions in the PIAS structure. Further, a portion of the N-terminal region of PIAS is required for the inhibiting STAT, therefore, such portions can be used to generate fusion proteins in methods for making antibodies and other diagnostic or therapeutic applications. Further still, regions of PIAS that show immunogenic structure, as well as other regions and domains, can readily be identified using various other methods known in the art, such as Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-

Wolf analysis. For example, it appears that the N-terminus of PIAS (e.g., the area located N-terminus of the zinc binding motif) to binds STAT. Fragments containing these residues may be particularly suited in generating specific classes of anti-PIAS antibodies.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective. Administration of a PIAS immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is PIAS or fragment thereof. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of PIAS can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin.

The antibody or fragment thereof of the invention may be labeled with a detectable marker or conjugated to a second molecule, such as a cytotoxic agent, and used for targeting the second molecule to an PIAS positive cell (Vitetta, E.S. et al., 1993, Immunotoxin therapy, in DeVita, Jr., V.T. et al., eds, Cancer: Principles and Practice of Oncology, 4th ed., J.B. Lippincott Co., Philadelphia, 2624-2636). Examples of cytotoxic agents include, but are not limited to ricin, doxorubicin, daunorubicin, taxol, ethiduim bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphteria toxin, *Pseudomonas* exotoxin (PE) A, PE40, ricin, abrin, and glucocorticoid and other chemotherapeutic agents, as well as radioisotopes. Suitable detectable markers include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, chemiluminescent compound, a metal chelator or an enzyme.

PIAS antibodies conjugated with toxic agents, such as ricin, as well as unconjugated antibodies are encompassed by the invention. Techniques for conjugating therapeutic agents to antibodies are well known (see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982)).

NUCLEIC ACID MOLECULES OF THE INVENTION

The present invention provides nucleic acid sequences encoding the STAT-binding proteins of the invention. The nucleic acid sequences can be DNA, RNA, DNA/RNA hybrid, and related molecules, nucleic acid molecules complementary to the PIAS coding sequence or a part thereof, and those which hybridize to the PIAS gene or to PIAS-encoding nucleic acids. Particularly preferred nucleic acid molecules will have a nucleotide sequence substantially identical to or complementary to the human or murine DNA sequences herein disclosed. Specifically contemplated are genomic DNA, cDNAs, ribozymes, and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases, whether derived from natural sources or synthesized. For example, antisense molecules can be RNAs or other molecules, including peptide nucleic acids (PNAs) or non-nucleic acid molecules such as phosphorothioate derivatives, that specifically bind DNA or RNA in a base pair-dependent manner. A skilled artisan can readily obtain these classes of nucleic acid molecules using the herein described PIAS sequences. For convenience, PIAS-encoding nucleic acid molecules will be referred to herein as PIAS-encoding nucleic acid molecules, PIAS genes, or PIAS sequences.

Embodiments of the PIAS-encoding nucleic acid molecules of the invention include primers, which allow the specific amplification of nucleic acid molecules of the invention or of any specific parts thereof, and probes that selectively or specifically hybridize to nucleic acid molecules of the invention or to any part thereof. The nucleic acid probes can be labeled with a detectable marker. Examples of a detectable marker include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Such labeled probes can be used to diagnose the presence of a PIAS protein as a means for detecting cells expressing a PIAS protein. Technologies for generating DNA and RNA probes are well known.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules that encode polypeptides other than PIAS. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated PIAS-encoding nucleic acid molecule.

The invention further provides fragments of the PIAS-encoding nucleic acid molecules of the present invention. As used herein, a fragment of a PIAS-encoding nucleic acid molecule refers to a small portion of the entire PIAS-encoding sequence. The size of the fragment will be determined by its intended use. For example, if the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming. Fragments of PIAS that are particularly useful as selective hybridization probes or PCR primers can be readily identified from the entire PIAS sequence using art-known methods.

Additionally, the invention provides vectors having the nucleic acid sequences above. Also provided are host-vector systems comprising vectors of the invention transfected into a compatible host cell and expressing PIAS (Sambrook *et al., Molecular Cloning* (1989).

The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a PIAS protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of a PIAS gene. Eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Any prokaryotic host can be used to express a PIAS-encoding DNA molecule, e.g., *E. coli*.

Transformation of appropriate cell hosts with a nucleic acid molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example,

Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing DNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

Successfully transformed cells, i.e., cells that contain a nucleic acid molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of a nucleic acid molecule of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

METHODS OF THE INVENTION

Further provided are methods for producing PIAS molecules of the invention.

These methods include recombinant methods and production methods known in the art such as growing cells containing any one of the host vector systems of the invention so as to produce the PIAS molecules in the host and recovering the protein so produced.

For example, a nucleic acid molecule is obtained that encodes a PIAS protein or a fragment thereof. The PIAS-encoding nucleic acid molecule is then preferably placed in an operable linkage with suitable control sequences, as described above, to generate an expression unit containing the PIAS-encoding sequence. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the PIAS protein. Optionally the PIAS protein is isolated from the

medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps may be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in an appropriate host. The construction of expression vectors that are operable in a variety of hosts is accomplished using an appropriate combination of replicons and control sequences. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with PIAS-encoding sequences to produce a PIAS protein.

Additionally, the invention provides methods for using the proteins of the invention.

For example, the invention provides methods for blocking (or inhibiting) the activity (e.g., DNA-binding activity) of STAT proteins (e.g., STAT1 or STAT3). In one embodiment, the method comprises contacting a PIAS molecule or portion thereof that bind a STAT protein (e.g. so that a PIAS/STAT complex is formed) so as to block the activity (e.g., DNA-binding activity) of the STAT protein so contacted. Further, STAT contacted with PIAS can prevent STAT from activating genes (gene transcription) controlled by STAT. These genes controlled by STAT include but are not limited to CFOS and Fc(γ) receptor.

The invention additionally provides methods for regulating an IFN-associated immune response mediated by STAT. The immune response includes an anti-viral response mediated by IFN, anti-tumor response mediated by IFN, and/or B and T cell responses mediated by IFN. In one embodiment of the invention, the method comprises contacting STAT positive cells (e.g., STAT1 positive cells) with a PIAS molecule (e.g., any of the PIAS1 proteins of the invention) and blocking the DNA-binding activity of STAT.

Blocking the DNA-binding activity of STAT prevents STAT from activating genes that regulates the IFN-associated immune response.

With respect to the methods of the invention, the most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the severity and course of the disease, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

For example, the interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Ferrite, E.J., et al. (Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey and Man. Cancer Chemother, Rep., 50, No.4, 219-244, May 1966).

Adjustments in the dosage regimen can be made to optimize the response. Doses can be divided and administered on a daily basis or the dose can be reduced proportionally depending upon the situation. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the specific therapeutic situation.

The following example is presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The example is not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

This example describes the identification of PIAS3.

PIAS1 which can specifically interact with STAT1 was cloned using the yeast two-hybrid assays (Taniguchi et al. (1995). The expressed sequence tag (EST) database was searched for other PIAS family members (GENBANK). The database search was done

with the Baylor College of Medicine Search Launcher. A human EST clone encoding a polypeptide related to the COOH-terminal portion of PIAS1 was identified. The name of this EST clone was HE6WCR27 (GenBank accession number H58757).

Using PIAS1 so cloned, a full-length cDNA of murine PIAS3 was obtained containing an open reading frame of 583 amino acids by screening a mouse thymus library with the human EST clone. Figure 1 shows the primary sequence and expression of PIAS3. In Figure 1a, the predicted amino acid sequence of mouse PIAS3 is shown. The four cysteine residues that are predicted to form a zinc finger are underlined. In Figure 1b, expression of PIAS3 mRNA in human tissues is shown. Human tissue blot was probed with human EST clone HE6WCR27 following manufacture's instructions.

PIAS3 protein contains a putative zinc-binding motif [C_2-$(X)_{21}$-C_2], a feature conserved in the PIAS family (Fig. 1A). Northern (RNA) blot analysis indicated that PIAS3 is widely expressed in various human tissues (Fig. 1B). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr.

FUNCTION OF PIAS3

To study the function of PIAS3 in vivo, a specific antiserum (anti-PIAS3c) was prepared to a recombinant fusion protein of glutathione S-transferase (GST) with the 79 COOH-terminal amino acid residues of PIAS3. This antiserum detected a protein with a molecular mass of about 68 kD, the predicted size of PIAS3, in both cytoplasmic and nuclear extracts of a number of human and murine cell lines.

To identify which STAT protein interacts with PIAS3, protein extracts were prepared from murine myeloblast M1 cells, which were untreated or treated with IL6. Proteins immunoprecipitated with anti-PIAS3c were analyzed by protein immunoblot with anti-STAT3. STAT3 was present in a PIAS3 immunoprecipitate from IL6 treated M1 cells but not in an immunoprecipitate from untreated M1 cells (Fig. 2A). A reblot of the filter with anti-PIAS3c showed that similar amounts of PIAS3 were present in each lane. IL6 stimulation can induce tyrosine phosphorylation of STAT1 as well as STAT3 (Sadowski et al. (1993)). The protein blot was therefore washed and reprobed with antibody to STAT1. STAT1 was not present in PIAS3 immunoprecipitates (Fig. 2A). Furthermore, PIAS3 was not found to be associated with STAT1 in a number of cell lines treated with interferon-γ. These results indicate that PIAS3 specifically interacts with STAT3.

Figure 2 shows the in vivo interaction of PIAS3 with STAT3. Figure 2a shows treatment with IL6 induced the interaction of PIAS3 with STAT3. Protein extracts from M1 cells, untreated (-) or treated with IL6 for 10 min (+), were subjected to immunoprecipitation (IP) with anti-PIAS3c. the blot was probed with anti-STAT. The same blot was then reprobed with anti-PIAS3c. The filter was washed and reprobed with anti-STAT1.

STAT3 can be activated by other cytokines in the IL6 family, such as ciliary neurotrophic factor (CNTF) and oncostatin M (OM) (Akira et al. (1994)). In human HepG2 cells, STAT3 was associated with PIAS3 in cells stimulated with CNTF or OM but not in untreated cells (Fig. 2B).

Figure 2b shows treatment with CNTF or OM induces the interaction of PIAS3 with STAT3. Protein extracts from human HepG2 cells, untreated or treated with CNTF or OM for 10 min, were subjected to immunoprecipitation with anti-PIAS3c. The blot was probed with anti-STAT3. Whole-cell extracts were prepared with lysis buffer containing 1% Brij, 50 mM tris (pH 8), 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM

phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), aprotinin (3 μg/ml0, pepstatin (1 μg/ml), and 0.1 mM sodium vanadate. The mixture was rotated at 4°C for 30 min and centrifuged at 13,000g for 5 min. The supernatant was used for immunoprecipitation with anti-PIAS3c (1:100 dilution). Immunoprecipitation and protein immunoblotting were done as described.

The effect of PIAS3 on the DNA-binding activity of STAT3 was tested. Nuclear extracts from HepG2 cells were prepared and analyzed in mobility gel shift assays, with a high-affinity STAT3-binding site as the probe (Zhong et al. (1994); Sadowski et al. (1993)). Treatment with IL6 induced the binding of three distinct gel shift complexes (Zhong et al. (1994); Sadowski et al. (1993)) corresponding to a STAT3-STAT3 homodimer, a STAT3-STAT1 heterodimer, and a STAT1-STAT1 homodimer (Fig. 3A).

A recombinant fusion protein of GST with PIAS3 (GST-PIAS3) was prepared and purified and added (in 20- to 200-ng quantities) to IL6-treated HepG2 nuclear extracts. GST-PIAS3 (100 ng) completely inhibited the DNA-binding activity of the STAT3-STAT3 homodimer and the STAT3-STAT1 heterodimer (Fig. 3A) but had no effect on the DNA-binding ability of the STAT1-STAT1 homodimer. As a control, GST alone did not inhibit the DNA-binding ability of any of the three complexes. A similar inhibitory effect of PIAS3 on the DNA-binding activity of STAT3 was observed in nuclear extracts prepared from IL6-treated M1 and MCF7 cell.

To further demonstrate the specific inhibitory effect of GST-PIAS3 on STAT3, the effect of GST-PIAS3 was tested on the DNA-binding activity of nuclear factor kappa B (NF-κB). Nuclear extracts prepared from untreated MCF7 cells or MCF7 cells treated with tumor necrosis factor-α (TNF-α) were analyzed by mobility gel shift analysis with

a NF-κB-binding site as the probe. TNF-α induced the formation of an NF-κB gel shift complex. The presence of either GST or GST-PIAS3 had no effect on the DNA-binding activity of NF-κB (Fig. 3B). These results demonstrate that PIAS3 can specifically inhibit the DNA-binding activity of STAT3.

Figure 3a/b shows the inhibition of the DNA-binding activity of STAT3 by PIAS3. Figure 3a provides electrophoretic mobility shift assays that were performed with nuclear extracts prepared from HepG2 cells with (+) or without (-) IL6 treatment in the absence of various amounts of either GST or GST-PIAS3 proteins (20 to 200 ng) as indicated. Mobility shift assays were done as described. The probe used is a high-affinity STAT3-binding site to which both STAT1 and STAT3 can bind (Zhong et al. (1995); Leveillard et al. (1993)). STAT-1, STAT1 homodimer; STAT3-3, STAT3 homodimer; STAT1-3, STAT1 and STAT3 heterodimer.

Figure 3b provides the same electrophoretic mobility shift assays Figure 3a, except that nuclear extracts were prepared from MCF7 cells with (+) or without (-) TNF-α treatment. The probe was derived from the NF-κB binding site in the promoter of the NF-κB inhibitor I-κB gene (Leveillard et al. (1993)). GST-PIAS3 was constructed by insertion of the cDNA into the Sal I and Not I cloning sites of pGEX4T-1. The concentration of GST-PIAS3 was estimated on 7% SDS-polyacrylamide gel electrophoresis with various dilutions of bovine serum albumin as the standard.

To test the effect of PIAS3 on STAT3-mediated gene activation, we transiently transfected HepG2 cells with expression vectors encoding STAT3 and FLAG-tagged PIAS3. Interleukin-6 can induce the association of PIAS3 with STAT3 in HepG2 cells. A luciferase reporter construct [(4X)IRF-1] containing four copies of the STAT-binding sequence from the interferon regulatory factor-1 (IRF-1) gene was used (Wen et al. (1995)). Cotransfection of STAT3 with (4X)IRF-1 resulted in about 20-fold stimulation of luciferase expression when cells were treated with IL6 (Fig. 4A). In the presence of

various amounts of PIAS3 (0.5 μg and 1 μg), STAT3-mediated induction of luciferase expression in response to IL6 stimulation was inhibited (Fig. 4A).

Luciferase assays were also performed in human embryonic 293 cells. Interferon-α (INF-α) stimulation can activate STAT3 in 293 cells (Wen et al. (1995)). Cells cotransfected with STAT3 and (4X)IRF-1 reporter construct showed a 150-fold increase of luciferase expression in response to INF-α (Fig. 4B). In the presence of PIAS3 (1 μg), however, the IFN-α induced, STAT3-dependent gene activation was almost completely inhibited.

PIAS3 (1 μg) had no such inhibitory effect on STAT1-mediated transcription activated in response to IFN-α (Fig. 4C). These results are in accord with the data that PIAS3 does not interact with STAT1 or inhibit its DNA-binding activity and indicate that PIAS3 is a specific inhibitor of STAT3-mediated gene activation.

Figure 4 shows the effect of PIAS3 on STAT-mediated gene activation. Panel A of Figure 4 shows inhibition of STAT3-mediated gene activation in response to IL6. HepG2 cells were transiently transfected with (4X)IRF-1 luciferase reporter construct together with an empty expression vector, STAT3, or various amount of FLAG-PIAS3 vectors, alone or in combination as indicated. Twenty-four hours after transfection, cultures were either left untreated (open columns) or treated with IL6 (10 ng/ml) for 6 hours (solid columns), and cell extracts were prepared and measured for luciferase activity.

Panel B shows inhibition of STAT3-mediated gene activation in response to IFN-α. Human 293 cells were transfected with (4X)IRF-1 luciferase reporter construct together with STAT3 or PIAS3 (or both) as indicated. Twenty-four hours after transfection, cells

were left untreated (open columns) or treated with IFN-α (5ng/ml) for 6 hours (solid columns), and luciferase activity was determined.

Panel C shows the effect of PIAS3 on STAT1-mediated gene activation. Panel C is the same as panel (B) except that STAT3 was replaced with STAT1 in cotransfection assays. FLAG-PIAS3 was constructed by insertion of the cDNA into the Sal I and Hind III sites of pCMV5-FLAG. HepG2 cells were transfected by modified calcium phosphate method. Cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 25-hydroxycholesterol (2.5 μg/ml) and were maintained at 350°C and 3% CO_2 for 3.5 hours during transfection. 293 cells were transfected by the calcium phosphate method (Shuai et al. (1994)). Data shown are taken from one representative experiment and was repeated at least three times. The relative luciferase units were corrected relative to the expression of β-galactosidase.

EXAMPLE 2

This example describes the identification, isolation, and characterization of PIAS1. PIASy, PIASxα, and PIASxβ are also described.

In this Example, the yeast two-hybrid method was used to identify additional proteins which can interact with STAT1 (Taniguchi et al. (1995)). By EST database searching and library screening, the murine PIAS1 as well as four related clones that encode putative new members of the PIAS family were identified (Meraz et al. (1996)).

The murine full length PIAS1 cDNA was obtained by sequencing EST clone #930725 (GENBANK). EST clone #785675 was used to screen a human testis library to obtain PIASxα and PIASxβ. EST clone #59244 was sequenced and identified as PIASy.

STAT1ß fused to LexA was used as a bait to screen a yeast interaction library prepared from human JY112 B cells [D. D. Chang, C. Wong, H. Smith, J. Liu, *JCB*, 138,1149 (1997)]. Fifty positive clones were identified from $3x10^6$ primary transformants. Of these clones, 40 were partial-length cDNAs encoding the COOH-terminal 313 amino acids of PIAS1. A longer cDNA clone which was obtained by screening a human K562 cDNA library was fused with EST clone #301840 to generate the full length human PIAS1 cDNA.

Figure 5 provides a sequence comparison of the PIAS family of proteins. The predicted amino-acid sequences of PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy are shown. h: human; m: mouse. Cysteine and histidine residues that are predicted to form a zinc finger (also referred to herein as the zinc binding motif) are shaded. The conserved acidic region is boxed. Dots indicate amino acid identity. The nucleotide sequences of each of these genes has been deposited in GenBank under the accession numbers hPIAS1: AF077951; mPIAS1: AF077950; hPIASxα: AF077953; hPIASxβ: AF077954; hPIASy: AF077952.

To test if PIAS1 is involved in regulating STAT1 activity, the effect of PIAS1 on STAT1-mediated gene activation by luciferase assays was examined. A luciferase reporter construct [(3x)Ly6] containing three copies of the STAT1-binding sequence from the murine Ly-6A/E gene was used (Shuai et al. Cell (1994); David et al. (1993)). Human 293 cells were transfected with expression vectors encoding FLAG-tagged STAT1 and PIAS1 together with [(3x)Ly6] in various combinations as indicated (Fig. 6A).

Panel A of Figure 6 provides Luciferase reporter assays. Human 293 cells were transiently transfected with [(3x)Ly6] luciferase reporter construct together with empty expression vector, FLAG-STAT1 or various amounts of FLAG-PIAS1 vectors, alone or in combination as indicated. Twenty four hours after transfection, cultures were either left untreated or treated with IFN-γ for 6 hours and cell extracts were prepared and

measured for luciferase activity (Promega). The relative luciferase units were corrected for relative expression of β-galactosidase. Calcium phosphate was used for transfection (Shuai et al. Science (1993)). Cells cotransfected with STAT1 and [(3x)Ly6] reporter construct showed a 35-fold increase of luciferase expression in response to IFN-γ. In the presence of an increasing amount of PIAS1, the STAT1-activated luciferase expression in response to IFN-γ stimulation was dramatically inhibited (Fig. 6A).

The expression of STAT1 and PIAS1 in these transfections was confirmed by Western blot analysis of the same extracts with anti-FLAG antibody (Fig. 6B). Panel B provides the Western blot analysis. Equal amounts of protein extracts from (A) were analyzed by immunoblot with anti-FLAG (Sigma). FLAG-PIAS1 was constructed by insertion of the murine PIAS1 cDNA into the Bgl II and Sal I sites of pCMV-FLAG vector.

In contrast, neither PIAS3 nor PIASx was able to inhibit the STAT1-mediated gene activation (Durbin et al (1996); Haque et al. (1995)). These results demonstrated that PIAS1 can inhibit STAT1-mediated gene activation.

Figure 7 demonstrates that PIAS1 inhibits the DNA binding activity of STAT1. A recombinant fusion protein of glutathione-S-transferase (GST) with PIAS1 (GST-PIAS1) was prepared and purified as well as GST-PIAS3 and GST-PIASxα. The effect of these fusion proteins on the DNA binding activity of STAT1 was tested.

Nuclear extracts from human Daudi B cells treated with IFN-∝ were incubated with GST or GST-PIASxα or GST-PIAS3 or GST-PIAS1 (15 to 75 ng). The mixtures were then analyzed by gel retardation assays, using the STAT1 DNA binding site as the probe (Fig. 7). GST-PIAS1 (45 ng) completely blocked the DNA binding activity of STAT1. In contrast, GST, GST-PIASxα and GST-PIAS3 had little effect on STAT1 binding. These results suggest that PIAS1 but not other PIAS molecules, can inhibit the DNA binding activity of STAT1.

Electrophoretic mobility shift analysis was performed as described (Wu et al. (1997)). The probe used is a high-affinity STAT1-binding binding site (Sadowski et al. (1993)). The concentration of GST fusion proteins were estimated on 7% SDS-polyacrylamide gel electrophoresis with various dilutions of bovine serum albumin as the standard. GST-PIASxα and GST-PIAS1 constructs were prepared by insertion of human PIASxα cDNA or murine PIAS1 cDNA into the EcoRI and Not I sites of p4T-1 (Pharmacia).

The specificity of protein-protein interactions may be lost when examined in vitro or when assayed under overexpression conditions. Therefore, PIAS-STAT interactions were analyzed in vivo. A specific antiserum (anti-PIAS1n) was prepared against a GST fusion protein containing 119 amino acid residues from the NH2-terminal region of PIAS1 (amino acid 50 to 168). This antibody specifically recognized a protein with the molecular weight of 78 kDa, the predicted size of PIAS1, in a number of human and murine cell lines tested (Shuai et al. (1996)).

It was determined whether PIAS1 is associated with or bound to STAT1 in vivo. Protein extracts prepared from human Daudi B cells untreated or treated with IFN-∝ for 15 min were used for immunoprecipitation with anti-PIAS1n. The immunoprecipitates were then washed and analyzed by Western blot with anti-STAT1 antibody. STAT1 was present in the PIAS1 immunoprecipitate from cells treated with IFN-α but not from untreated cells (Figure 8A). These results suggest that PIAS1 is associated with STAT1 in vivo and that the PIAS1-STAT1 interaction is dependent on ligand stimulation.

Since IFN-α also activates STAT2 (Haspel et al. (1996)), it was determined whether PIAS1 could interact with STAT2 upon IFN-α stimulation. In panel A of Figure 8 PIAS1 interacts with STAT1 in vivo. Protein extracts from Daudi cells untreated (-) or treated (+) with IFN-γ for 15 minutes were prepared and used for immunoprecipitation with anti-PIAS1n. Immunoprecipitates were then analyzed on SDS-PAGE and the blot

was probed anti-STAT1. The filter was washed and reprobed with anti-PIAS1 (lower panel).

In panel B of Figure 8 PIAS1 does not interact with STAT2. Daudi cells were treated with IFN-γ for the times indicated. Whole cell extracts were prepared and one half of these extracts were used for immnunoprecipitation with anti-PIAS1n (left panel). Immunoprecipitation was carried out as described in (A) and the filter was probed with anti-STAT1 and anti-STAT2. The same filter was washed and reprobed with anti-PIAS1n (lower panel). The other half of protein extracts were subjected to immunoprecipitation with anti-STAT1 (right panel). The filter was probed with a mixture of anti-STAT1 and anti-STAT2 antibodies. p-STAT1: phosphorylated STAT1.

Human Daudi B cells were untreated or treated with IFN-α for various time periods. Protein extracts were prepared and subjected to immunoprecipitation with anti-PIAS1n followed by immunoblot with both anti-STAT1 and STAT2 antibodies. While STAT1 was associated with PIAS1 in IFN-treated cells, STAT2 was absent in PIAS1 immunoprecipitates (Fig. 8B).

In contrast, STAT2 was found to be present in STAT1 immunoprecipitates (Fig. 8B). This is consistent with the fact that upon IFN-∝ stimulation, a fraction of STAT1 and STAT2 proteins can form heterodimers (Kim et al. (1996)). These results further suggest that PIAS1 specifically interacts with STAT1 but not STAT2.

Panel C of Figure 8 provides a Western blot analysis. HepG2 cells were untreated or treated with IL6 or IFN-γ for 15 min and protein extracts were prepared and analyzed by immunoblot with anti-STAT1, or anti-pSTAT1 (NEB), or anti-STAT3 (Santa Cruz, California), or anti-pSTAT3 (NEB) as indicated.

In panel D of Figure 8 PIAS1 interacts with STAT1 but not STAT3. Protein extracts from (C) were immunoprecipitated with anti-PIAS1n. The precipitates were subjected to electrophoresis and the filter was blotted with anti-STAT1 (left panel). The same filter was washed and reprobed with anti-STAT3 (left panel) or reprobed with anti-PIAS1n (lower panel). (E) Same as (D) except that anti-PIAS3c was used for immunoprecipitation. The filter was probed with anti-STAT3 (left panel) or anti-STAT1 (right panel) or PIAS3c (lower panel). Immunoprecipitation analysis was performed as described (Durbin et al. (1996)).

PIAS3 is a specific inhibitor of STAT3 signaling (Durbin et al. (1996)). To examine the specificity of PIAS-STAT interactions, in vivo co-immunoprecipitation analysis was carried out with protein extracts prepared from human HepG2 cells untreated or treated with IFN-γ or IL6.

IFN-γ treatment activates STAT1, but not STAT3, in HepG2 cells--as shown by immunoblot analysis with antisera that can specifically recognize tyrosine phosphorylated STAT1 or STAT3 (Fig. 8D). IL6 treatment strongly induces the tyrosine phosphorylation of STAT3 but only weekly stimulates phosphorylation of STAT1 in HepG2 cells. Samples of the same protein extracts were subjected to immunoprecipitation analysis with anti-PIAS1n or anti-PIAS3c [an antiserum against the COOH-terminal 79 amino acid residues of PIAS3 (Durbin et al. (1996))]. The immunoprecipitates were then analyzed by protein blot with anti-STAT1 or anti-STAT3.

PIAS1 was found to be associated with STAT1 but not STAT3 (Fig. 8D). Since the activation of STAT1 by IL6 was weak, the association of PIAS1 with STAT1 in IL6 treated HepG2 cells was observed only when the blot was overexposed. In contrast, PIAS3 was found to be associated with STAT3, but not STAT1 (Fig. 8E). These experiments further suggest that individual PIAS molecules display specificity for STATs in vivo.

Upon IFN stimulation, STAT1 becomes tyrosine phosphorylated on a single tyrosine residue Tyr-701. This phosphorylation is required for the dimerization, nuclear translocation and DNA binding activity of STAT1 (Shuai et al. Science (1993); Shuai et al. Cell (1994)). Since PIAS1 is associated with STAT1 only in ligand-stimulated cells, it was determined whether IFN-induced tyrosine phosphorylation of STAT1 is required for the in vivo PIAS1-STAT1 interaction.

Two stable cell lines, Ctyr and C91, derived from U3A cells which do not express STAT1 protein (Muller et al. (1993)) were used for co-immunoprecipitation analysis. C91 and Ctyr cell lines were established by complementing U3A cells with the wild type STAT1 and a mutant STAT1 (Tyr-701->Phe), respectively (Shuai et al. Science (1993)). Phosphotyrosine blot analysis confirmed that the T701F STAT1 mutant protein was not tyrosine phosphorylated in respond to IFN-γ stimulation (Fig. 9A).

Figure 9 shows that phosphorylation on Tyr-701 of STAT1 is required for PIAS1-STAT1 interaction. U3A cells complemented with STAT1 or STAT1(Tyr-701->Phe) mutant protein were established as described (Darnell et al. (1994)). Cells were untreated or treated with IFN-γ for 15 minutes and protein extracts were prepared. In panel A of figure 9 immunoprecipitation was performed with anti-STAT1 followed by blotting with a specific phosphotyrosine antiserum [anti-pTyr (Transduction Laboratories, Lexington, KY)]. The same filter was washed and reprobed with anti-STAT1 (lower panel).

In panel B of Figure 9 immunoprecipitation was performed with anti-PIAS1n antiserum and blotted with anti-STAT1. The filter was washed and reprobed with anti-PIAS1 (lower panel). U3A and U3A-derived cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 10% CO_2.

STAT1 was clearly co-immunoprecipitated by anti-PIAS1 from C91 cells treated with IFN-γ (Fig. 9B). In contrast, anti-PIAS1 antibody failed to co-immunoprecipitate the T701P STAT1 mutant protein from Ctyr cells. These results suggest that IFN-induced phosphorylation on Tyr-701 of STAT1 is required for PIAS1-STAT1 interaction.

The identification of a family of PIAS molecules and the striking specific in vivo association between individual PIAS and STAT proteins strongly suggest the possible involvement of a specific PIAS inhibitor in every STAT signaling pathway. While not wishing to be bound by any specific theory, since PIAS molecules do not contain phosphotyrosine binding domains such as SH2 or PTB (Cohen et al. (1995); Pawson et al. (1997)), it seems likely that tyrosine phosphorylation of STATs may induce a protein conformational change, resulting the exposure of the PIAS binding domain.

PIAS molecules contain a conserved putative zinc binding motif which is present in many proteins including transcription factors. Interestingly, a N-terminal truncated mutant PIASxB protein (from amino acid 134 to 621), Miz1, was recently shown to interact with a homeobox DNA binding protein Msx2 (Wu et al. (1997)). In addition, Miz1 was shown to have sequence specific DNA binding activity (Wu et al. (1997)). Thus, a PIAS molecule may play a dual functional role of inhibiting the expression of genes containing STAT binding sites while activating another distinct set of genes.

In summary, a family of PIAS (protein inhibitor of activated STAT) proteins were isolated. PIAS1, but not other PIAS molecules, blocked the DNA binding activity of STAT1 and inhibited STAT1-mediated gene activation in response to interferon (IFN). Co-immunoprecipitation analysis showed that PIAS1 was associated with STAT1 but not STAT2 or STAT3 following ligand stimulation. The in vivo PIAS1-STAT1 interaction requires phosphorylation of STAT1 on Tyr-701. These results demonstrated the specificity of PIAS-STAT interaction and suggest that there may exist a specific PIAS inhibitor in every STAT signaling pathway.

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What is claimed is:

1. A purified PIAS molecule which specifically recognizes and binds a STAT protein thereby blocking the DNA-binding activity of STAT and regulating the activity of said STAT protein.

2. The isolated PIAS molecule of claim 1 which binds STAT3 but not STAT1.

3. The protein of claim 2 designated murine PIAS3 and having the sequence shown in Figure 5.

4. The protein of claim 2 designated human PIAS3 and having the sequence shown in Figure 5.

5. The protein of claim 2, wherein the STAT3 is a homodimer.

6. The protein of claim 2, wherein the STAT3 is a heterodimer.

7. The protein of claim 2, wherein the STAT3 is STAT3(α).

8. The protein of claim 2, wherein the STAT3 is STAT3-ß.

9. The protein of claim 1 which binds STAT1 but does not bind STAT3.

10. The protein of claim 9 designated human PIAS1 and having the sequence shown in Figure 5.

11. The protein of claim 9 designated murine PIAS1 and having the sequence shown in Figure 5.

12. The protein of claim 1 designated human PIASxα and having the sequence shown in Figure 5.

13. The protein of claim 1 designated human PIASxβ and having the sequence shown in Figure 5.

14. The protein of claim 1 designated human PIASy and having the sequence shown in Figure 5.

15. The protein of claim 9, wherein the STAT1 is a homodimer.

16. The protein of claim 9, wherein the STAT1 is a heterodimer.

17. The protein of claim 9, wherein the STAT1 is STAT1(α).

18. The protein of claim 9, wherein the STAT1 is STAT1-ß.

19. A nucleic acid sequence encoding the protein of claim 1 or 9.

20. The nucleic acid sequence of claim 19 that is DNA.

21. The nucleic acid sequence of claim 20, wherein the DNA is cDNA.

22. The nucleic acid sequence of claim 19 that is RNA.

23. A nucleic acid sequence that recognizes and binds the nucleic acid sequence of claim 19.

24. A vector having the nucleic acid molecule of claim 19.

25. A host-vector expression system comprising the vector of claim 24 transfected into a compatible eucaryotic host cell.

26. A method for producing a PIAS molecule comprising growing the host vector expression system of claim 25 so as to produce the protein in the host and recovering the PIAS molecule so produced.

27. A method for blocking the DNA-binding activity of a STAT protein comprising contacting a PIAS molecule with STAT so as to block the DNA-binding activity of the STAT protein so contacted.

28. The method of claim 27, wherein the PIAS molecule is PIAS1.

29. The method of claim 27, wherein the PIAS molecule is PIAS3.

30. The method of claim 27, wherein the PIAS molecule is PIASxα.

31. The method of claim 27, wherein the PIAS molecule is PIASxβ.

32. The method of claim 27, wherein the PIAS molecule is PIASy.

33. The method of claim 27, wherein the STAT protein is STAT1.

34. The method of claim 27, wherein the STAT protein is STAT3.

FIG. 1A

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MVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTL   61
GPSDLSLLSLPPGTSPPVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTP  122
QQLQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLP  183
PTKNGAEPRGPAVRSTSHPWLDSQPLSPTPSLLIGHLSLDGITPCPCLVRQLTAGTLLQKL  244
RAKGIRNPDHSRALIKEKLTADPDSEVATTSLPGVTHVPARKMRLTVPCRALTCAHLQSFD  305
AALYLQMNEKKPTWTCPVCDKKAPYESLIIDGLFMEILNSCSDCDEIQFMEDGSWCPMKPK  366
KEASEVCPPPGYGLDGLQYSAVQEGIQPESKKRVEVIDLTIESSSDEEDLPPTKKHCSPTS  427
AAIPALPGSKGALTSGHQPSSVLRSPAMGTLGSDFLSSLPVHEYPPAFPLGADIQGLDLFS  488
FLQTESQQYGPSVIISLDEQDTLGHFFQYRGTPSHFLGPLAPTLGSCHGSSTPAPPPGRVS  549
SIVAPGSSLREGHGGPLPSGPSLTGCRSDVISLD
```

FIG. 1B

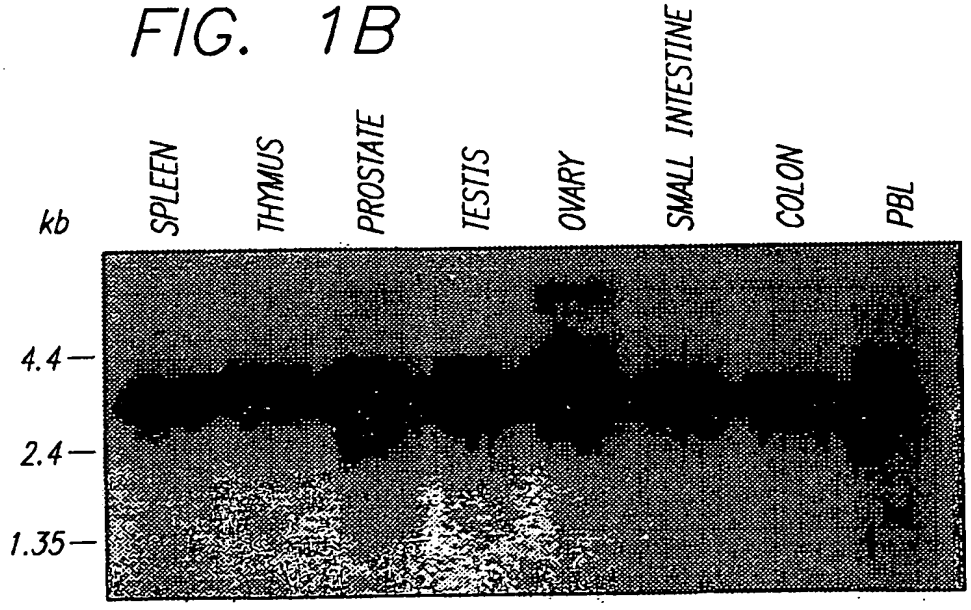


FIG. 2A

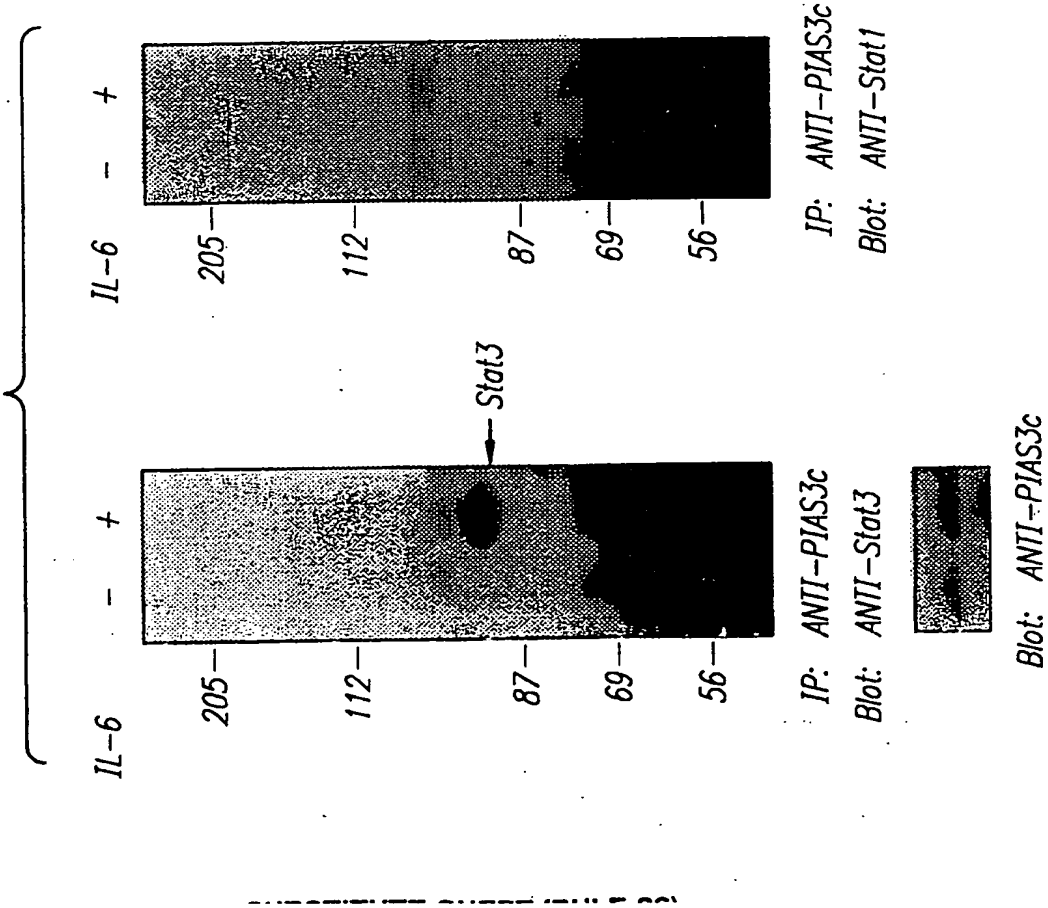

IL-6
- +
205
112
87
69
56
Stat3
IP: ANTI-PIAS3c
Blot: ANTI-Stat3
IL-6
- +
205
112
87
69
56
IP: ANTI-PIAS3c
Blot: ANTI-Stat1
Blot: ANTI-PIAS3c

FIG. 2B

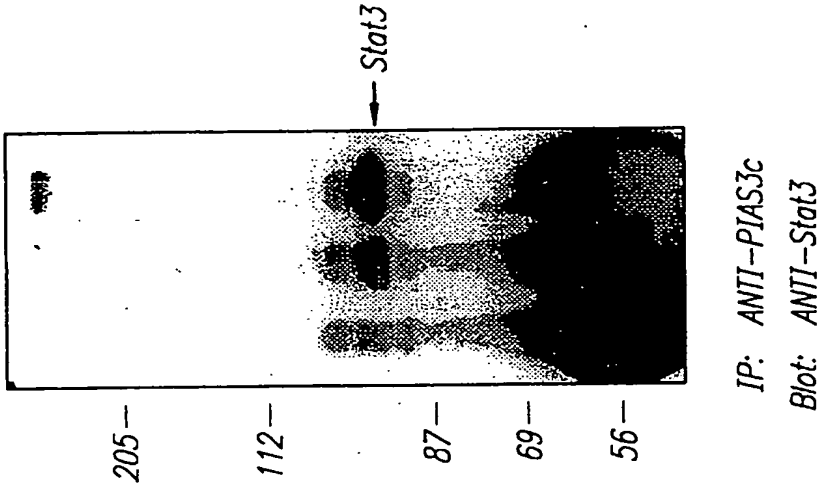
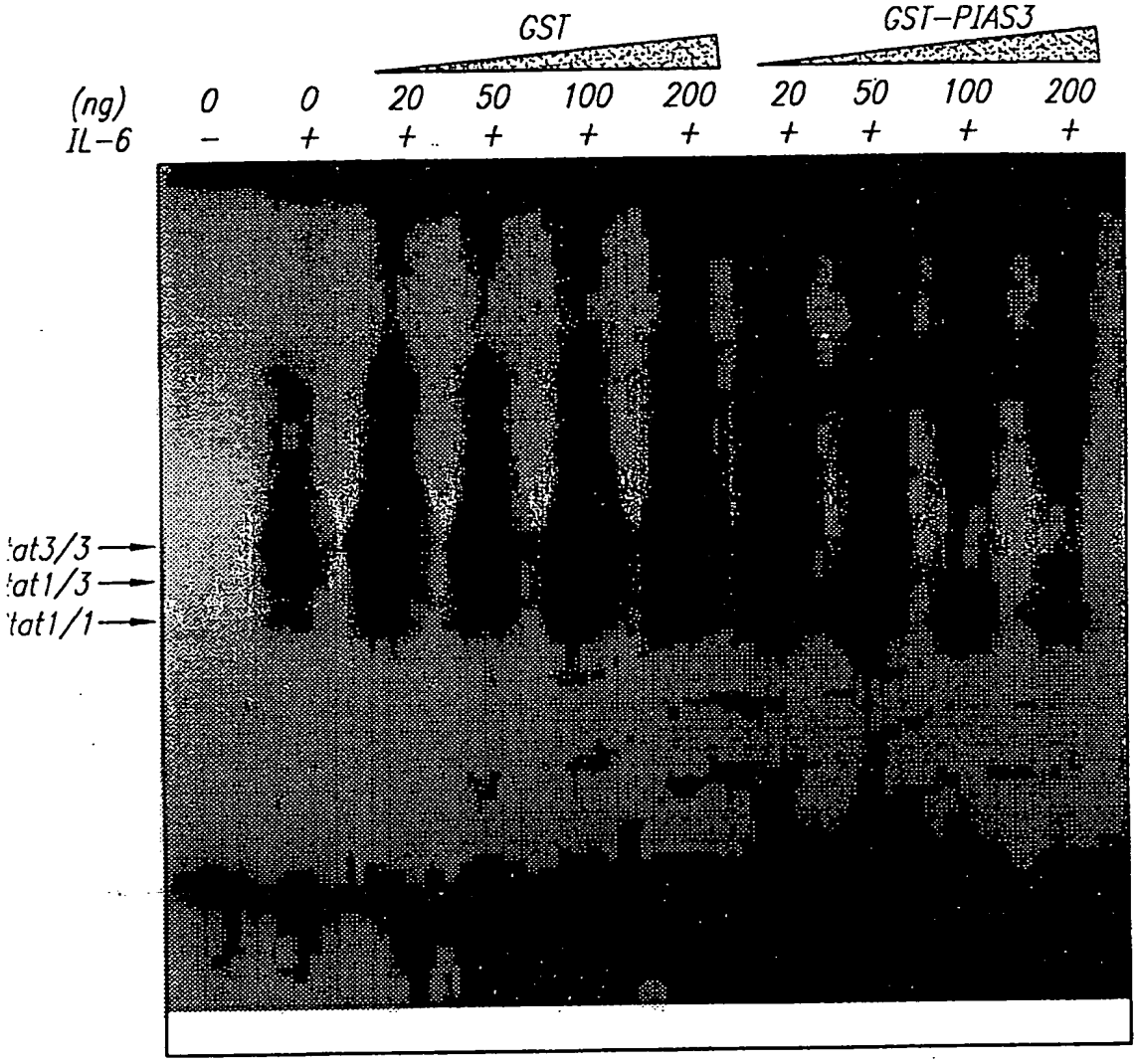

- CNTF OM
205
112
87
69
56
Stat3
IP: ANTI-PIAS3c
Blot: ANTI-Stat3

FIG. 3A


GST
GST–PIAS3
(ng) 0 0 20 50 100 200 20 50 100 200
IL–6 – + + + + + + + + +
tat3/3
tat1/3
tat1/1

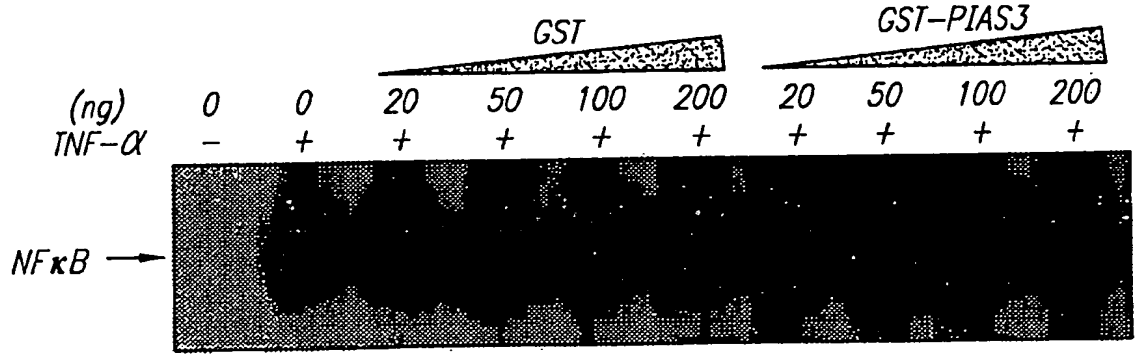

GST
GST–PIAS3
(ng) 0 0 20 50 100 200 20 50 100 200
TNF–α – + + + + + + + + +
NFκB

FIG. 3B

FIG. 4A

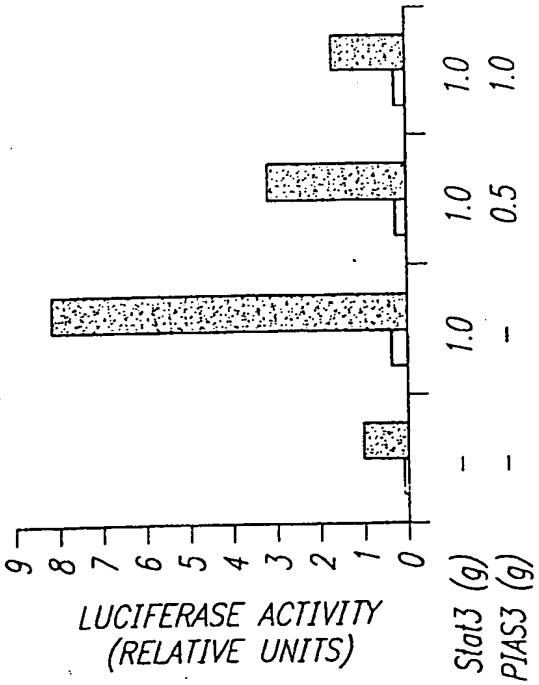


FIG. 4B

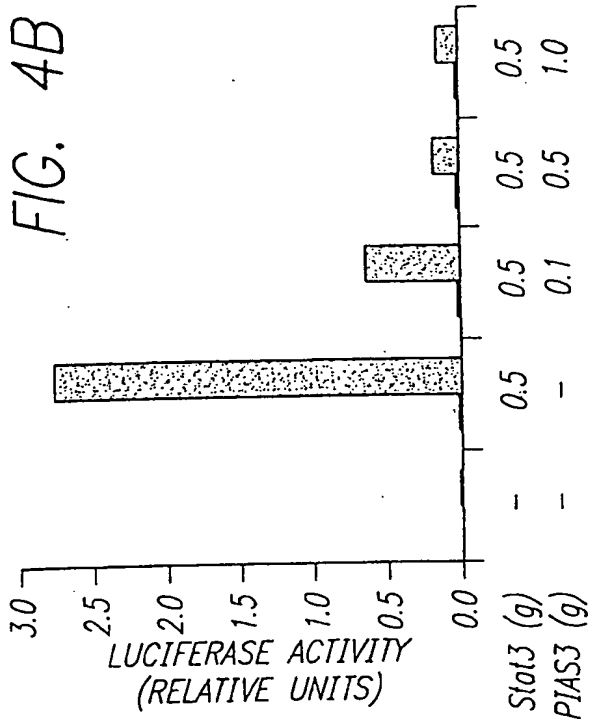


FIG. 4C

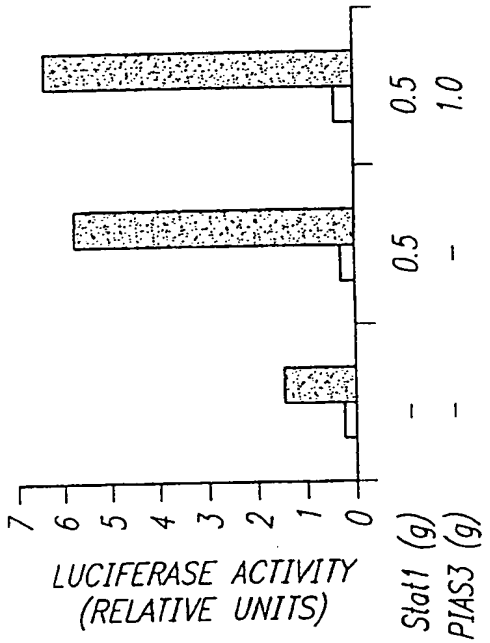


FIG. 5-1

```
hPIAS1     MADSAELKQMVMSL RVSELQVLLGYAGRN KHGRKHELLTKALHL LKAGCSPAVQMKIKE LYRRRFPQKIMTPAD LSIPNVH-------S   82
mPIAS1     ..............  ..............  ..............  ..............  ..............  ..............   82
mPIAS3     ....F ..........F.... .S.......A..... ..SS.A.S....... .......R.TLG.S. ..L-----LSLPPGT   75
hPIASx α   ...FE..RN..S F ..........F.... .S....D..MR.... ...S......I..R. .....Y.RTLEGLS. ..TIKSSVFSLDGG.   89
hPIASx β   ...FE..RN..S.F ..........F.... .S....D..MR.... ..S.......I..R. .....Y.RTLEGLS. ..TIKSSVFSLDGG.   89
hPIASy     MA.ELV.A.N....F ...D..M...FV..S .S.L....V.R..Q. VQFD...ELFK.... ..ET.YAK.NSE..P ---------QPHRPL   81

hPIAS1     SPMPATLSPS---TI PQLTYDGHPASSPLL PVSLLGPKHKLELPH LTSALHPVHPDIKLQ KLPFYDLLDELIKPT SLASDNSQRFRETCF  169
mPIAS1     ....P.....---.. ..............  .........E.....  ..............  ..............  ..............  169
mPIAS3     ..------------- -------------- -------------- ------.....VTMK P....EVYG...R.. T...TS....E.AH.  116
hPIASx α   ..VEPD.AVAGIHSL .STSVTP.SP...VG S.L.QDT.PTF.MQQ PSPPIP.....VQ.K N.....V..V..... ..VQSSI...Q.KF.  179
hPIASx β   ..VEPD.AVAGIHSL .STSVTP.SP...VG S.L.QDT.PTF.MQQ PSPPIP.....VQ.K N.....V..V..... ..VQSSI...Q.KF.  179
hPIASy     D.LTMHSTYDRAGAV .RTPLA.PNIDY.V. YGKY.NGLGR.---- ---PAKTLK.EVR.V ....FNM....L... E.VPQ.NEKLQ.SPC  164

hPIAS1     AFALTPQQVQQISSS MD-ISGTKCDFTVQV QLRFCLSETSCPQED HFPPNLCVKVNTKPC SLPGYLPPTKNGVEP KRPSRPINITSLVRL  258
mPIAS1     ..............  ..-...........  ..............  ..............  ..............  ..............  258
mPIAS3     T.......L...LT. REVLP.A...Y.I.. ......C.......  Y.....F....G.L. P...........A.. RG.A--VRS..HPW.  204
hPIASx α   I........RE.CI. R.FLP.GRR.Y.... ...L..A.......  NY.NS..I...G.LF P....A..P...I.Q ...G..L.......  269
hPIASx β   I........RE.CI. R.FLP.GRR.Y.... ...L..A.......  NY.NS..I...G.LF P....A..P...I.Q ...G..L.......  269
hPIASy     I.....R..EL.RKF QGMQP.V.---A... V..I.Y.D.......  QY...IA....HSY. .V...Y.SN.P.... ...C....L.H.MY.  251

hPIAS1     STTVPNTMC-SWTAE IGR----NYSMAVYL VKQLSSTVLLQRLRA KGIRNPDHSRALIKE KLTADPDSEIATTSL RVSLLCPLGKMRLTI  343
mPIAS1     .......IVV.....  ..----.........  ..............  ..............  .....S.........  ..............  344
mPIAS3     DS---QPLSPTPSLL ..HLSLDGITPCPC. .R..TAGT...K...  ..............  .........V.....  PGVTHV.AR.....V  291
hPIASx α   .SA...QISI..AS. ..K----....S...  VR..T.AM.....KM  ..............  ..............  ....M.........  355
hPIASx β   .SA...QISI..AS. ..K----....S...  VR..T.AM.....KM  ..............  ..............  ....M.........  355
hPIASy     .SAT-.RITVT.GNY -.K----S..V.L..  VR..T.SE.....KT I.VKH.ELCK..V..  ..RL.........GV  ....I...V....SV  335
```

FIG. 5-2

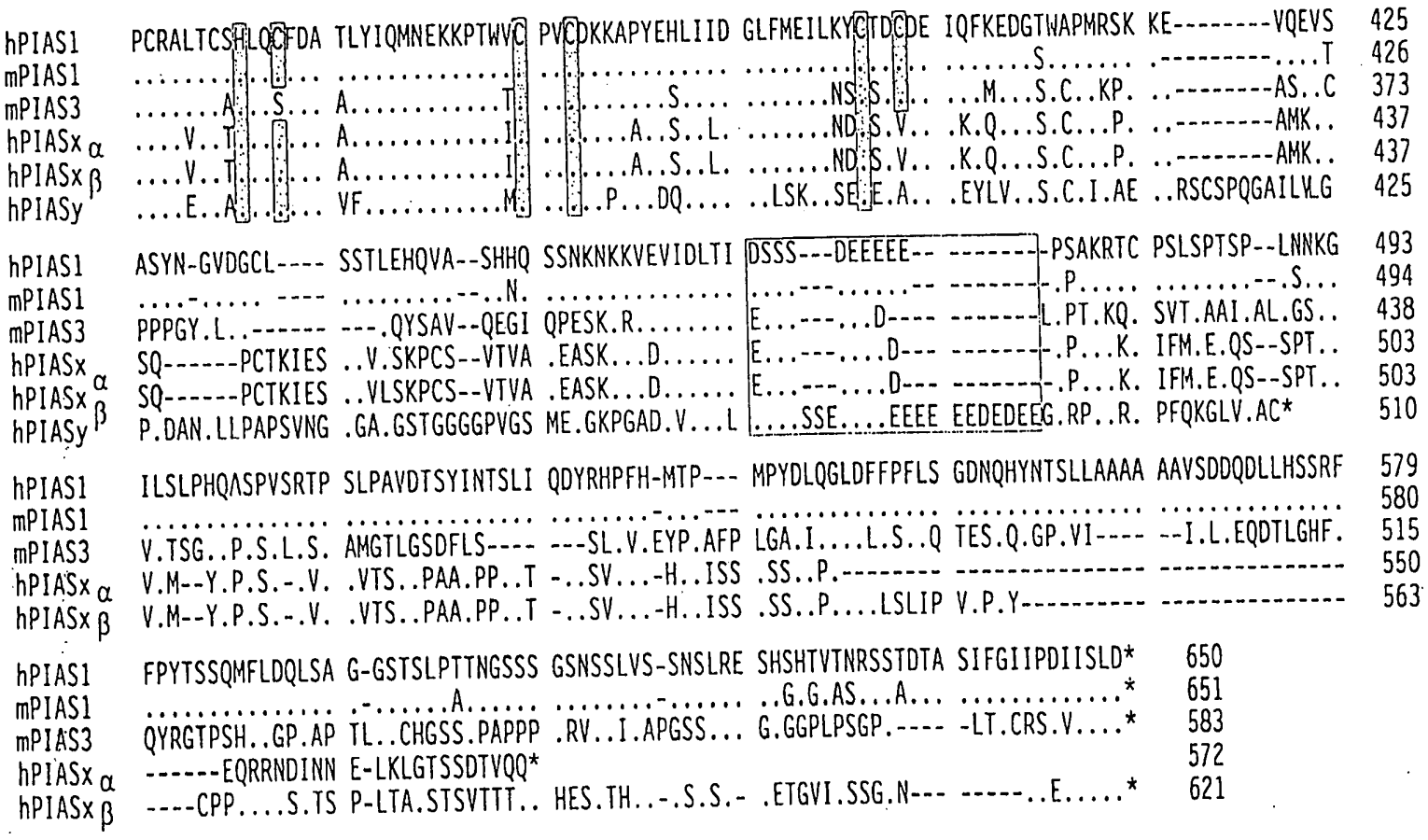

hPIAS1
mPIAS1
mPIAS3
hPIASx α
hPIASx β
hPIASy
425
426
373
437
437
425
493
494
438
503
503
510
579
580
515
550
563
650
651
583
572
621

FIG. 5-3

1/1 31/11
atg gtg atg agt ttc cgg gtg tct gag ctc cag gtg ctt ctt ggc ttt gct ggc cgg aac
M V M S F R V S E L Q V L L G F A G R N
61/21 91/31
aag agt gga cgg aag cac gag ctc ctg gcc aag gct ctg cac ctc ctg aag tcc agc tgt
K S G R K H E L L A K A L H L L K S S C
121/141 151/51
gcc cct agt gtc cag atg aag atc aaa gag ctt tac cga cga cgc ttt ccc cgg aag acc
A P S V Q M K I K E L Y R R R F P R K T
181/61 211/71
ctg ggg ccc tct gat ctc tcc ctt ctc tct ttg ccc cct ggc acc tct cct cct gtg cac
L G P S D L S L L S L P P G T S P P V H
241/81 271/91
cct gat gtc acc atg aaa cca ttg ccc ttc tat gaa gtc tat ggg gag ctc atc cgg ccc
P D V T M K P L P F Y E V Y G E L I R P
301/101 331/111
acc acc ctt gca tcc act tct agc cag cgg ttt GAG GAA GCG CAC TTT ACC TTT GCC CTC
T T L A S T S S Q R F E E A H F T F A L
361/121 391/131
ACA CCC CAG CAA GTG CAG CAG ATT CTT ACA TCC AGA GAG GTT CTG CCA GGA GCC AAA TGT
T P Q Q V Q Q I L T S R E V L P G A K C
421/141 451/151
GAT TAT ACC ATA CAG GTG CAG CTA AGG TTC TGT CTC TGT GAG ACC AGC TGC CCC CAG GAA
D Y T I Q V Q L R F C L C E T S C P Q E
481/161 511/171
GAT TAT TTT CCC CCC AAC CTC TTT GTC AAG GTC AAT GGG AAA CTG TGC CCC CTG CCG GGT
D Y F P P N L F V K V N G K L C P L P G
541/181 571/191
TAC CTT CCC CCA ACC AAG AAT GGG GCC GAG CCA AGA GGC CCA GCC GCC CCA TCA ACA TCA
Y L P P T K N G A E P R G P A A P S T S
601/201 631/211
CAC CCC TGG CTC GAC TCT CAG CCA CTG TTC CCA ACA CCA TTG TGG TCA ATT GGT CAT CTG
H P W L D S Q P L F P T P L W S I G H L
661/221 691/231
AGT TCG GAC GGA ATT ACT CCT TGT CTG TGT ACC TtG GTG AGG CAG TTG ACT GCA GGA ACC
S S D G I T P C L C T L V R Q L T A G T
721/241 751/251
CTT CTA CAA aaa ctc aga gca aag ggt atc cgg aac cca gac cac tcg cgg gca ctg atc
L L Q K L R A K G I R N P D H S R A L I
781/261 811/271
aag gag aaa ttg act gct gac cct gac agt gag gtg gcc act aca agt ctt ccg ggt gtc
K E K L T A D P D S E V A T T S L P G V
841/281 871/291
act cat gtg ccc gct agG aag atg cgc ctg act gtc cct tgt cgt gcc ctc acc tgc gcc

```
T  H  V  P  A  R  K  M  R  L  T  V  P  C  R  A  L  T  C  A
901/301                                931/311
cac ctg cag agc ttc gat gct gcc ctt tat cta cag atg aat gag aag aag cct aca tgg
H  L  Q  S  F  D  A  A  L  Y  L  Q  M  N  E  K  K  P  T  W
961/321                                991/331
aca tgt cct gtg tgt gac aag aag gct ccc tat gaa tct ctt atc att gat ggt tta ttt
T  C  P  V  C  D  K  K  A  P  Y  E  S  L  I  I  D  G  L  F
1021/341                               1051/351
atg gag att ctt agt tcc tgt tca gat tgt gat gag atc caa ttc atg gaa gat gga tcc
M  E  I  L  S  S  C  S  D  C  D  E  I  Q  F  M  E  D  G  S
1081/361                               1111/371
tgg tgc cca atg aaa ccc aag aag gag gca tct gag gtt tgc ccc ccg cca ggg tat ggg
W  C  P  M  K  P  K  K  E  A  S  E  V  C  P  P  P  G  Y  G
1141/381                               1171/391
ctg gat ggc ctc cag tac agc cca ggt cca ggg ggg aga tcc atc gag aat aag aag aag
L  D  G  L  Q  Y  S  P  G  P  G  G  R  S  I  E  N  K  K  K
1201/401                               1231/411
gtc gaa gtt att gac ttg aca ata gaa agc tca tca gat gag gag gat ctg ccc cct acc
V  E  V  I  D  L  T  I  E  S  S  S  D  E  E  D  L  P  P  T
1261/421                               1291/431
aag aag cac tgt tct gtc acc tca gct gcc atc ccg gcc cta cct gga agc aaa gga gtc
K  K  H  C  S  V  T  S  A  A  I  P  A  L  P  G  S  K  G  V
1321/441                               1351/451
ctg aca tct ggc cac cag cca tcc tcg gtg cta agg agc cct gct atg ggc acg ttg ggt
L  T  S  G  H  Q  P  S  S  V  L  R  S  P  A  M  G  T  L  G
1381/461                               1411/471
ggg gat ttc ctg tcc agt ctc cca cta cat gag tac cca cct gcc ttc cca ctg gga gcc
G  D  F  L  S  S  L  P  L  H  E  Y  P  P  A  F  P  L  G  A
1441/481                               1471/491
gac atc caa ggt tta gat tta ttt tca ttt ctt CAG ACA GAG AGT cag cac tat ggc ccc
D  I  Q  G  L  D  L  F  S  F  L  Q  T  E  S  Q  H  Y  G  P
1501/501                               1531/511
tct gtc atc atc tca cta gat gaa cag gat gcc ctt ggc cac ttc tTc cag tac cga ggg
S  V  I  I  S  L  D  E  Q  D  A  L  G  H  F  F  Q  Y  R  G
1561/521                               1591/531
acc cct tct cac ttt ctg ggc cca ctg gcc ccc acg ctg ggg agc tcc cac tgC agc gcc
T  P  S  H  F  L  G  P  L  A  P  T  L  G  S  S  H  C  S  A
1621/541                               1651/551
act ccg gcg ccc cct cct ggC cgt gtc agc agc att gtg gcc cct ggg ggg gcc ttg agg
T  P  A  P  P  P  G  R  V  S  S  I  V  A  P  G  G  A  L  R
1681/561                               1711/571
gag ggg cat gga gga ccc ctg ccc tca ggt ccc tct TTg act ggc tgt cgg tca gac atc
E  G  H  G  G  P  L  P  S  G  P  S  L  T  G  C  R  S  D  I
1741/581
att tcc ctg gac tga
I  S  L  D  *
```

FIG. 5—4

FIG. 6A

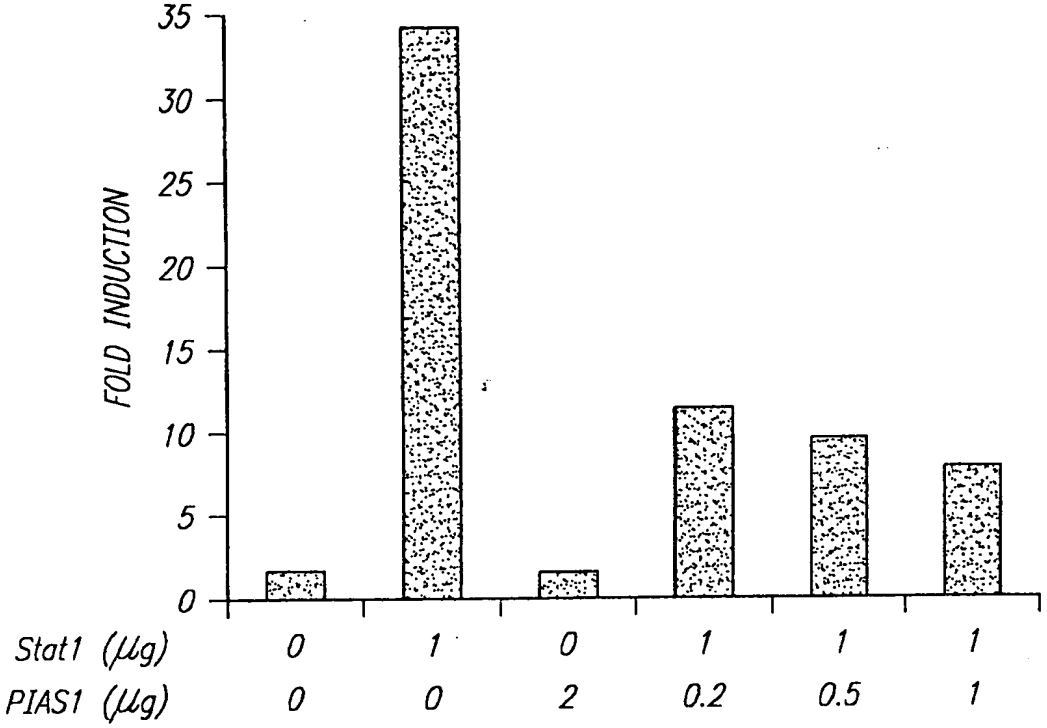
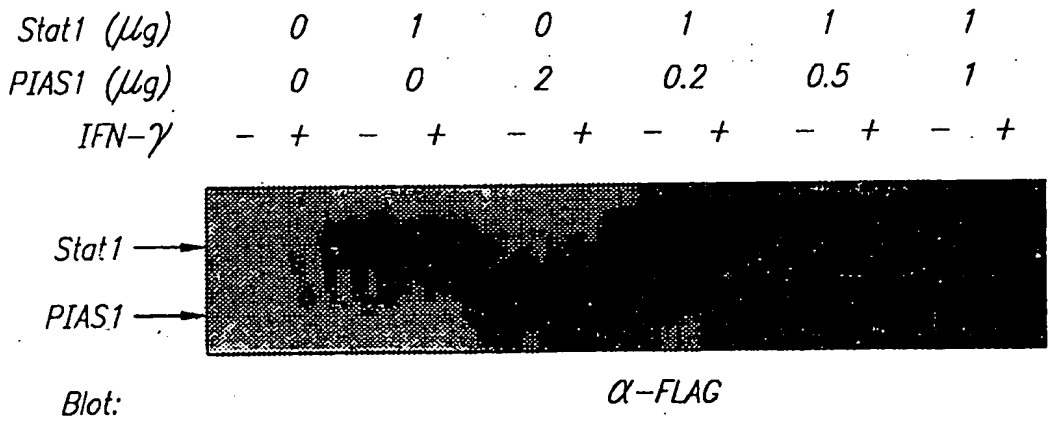

FOLD INDUCTION
35
30
25
20
15
10
5
0
Stat1 (μg) 0 1 0 1 1 1
PIAS1 (μg) 0 0 2 0.2 0.5 1

FIG. 6B


Stat1 (μg) 0 1 0 1 1 1
PIAS1 (μg) 0 0 2 0.2 0.5 1
IFN-γ - + - + - + - + - + - +
Stat1
PIAS1
Blot:
α-FLAG

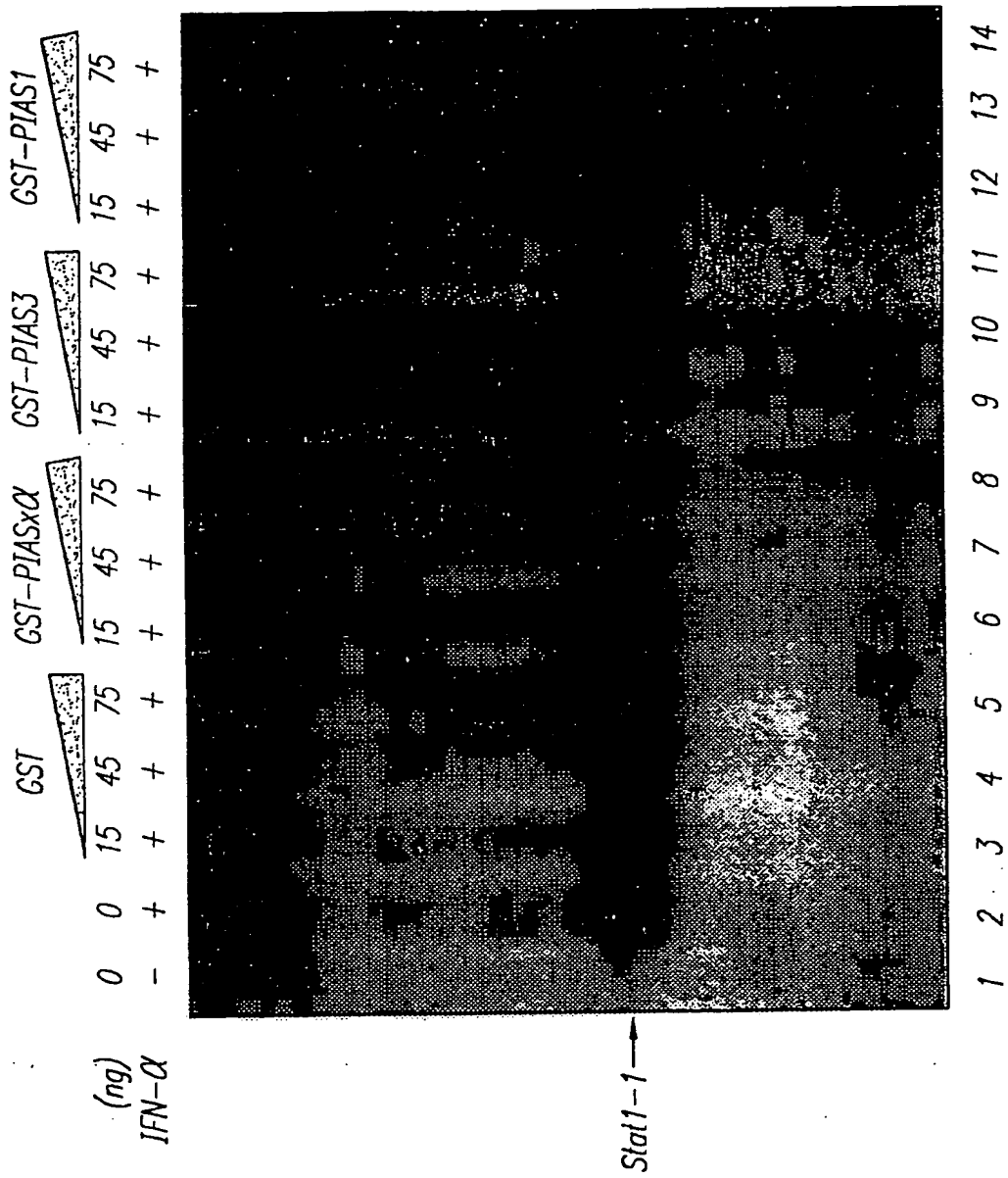

(ng) IFN-α
0 0
– +
GST
15 45 75
+ + +
GST-PIASxα
15 45 75
+ + +
GST-PIAS3
15 45 75
+ + +
GST-PIAS1
15 45 75
+ + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Stat1-1

FIG. 7

FIG. 8A

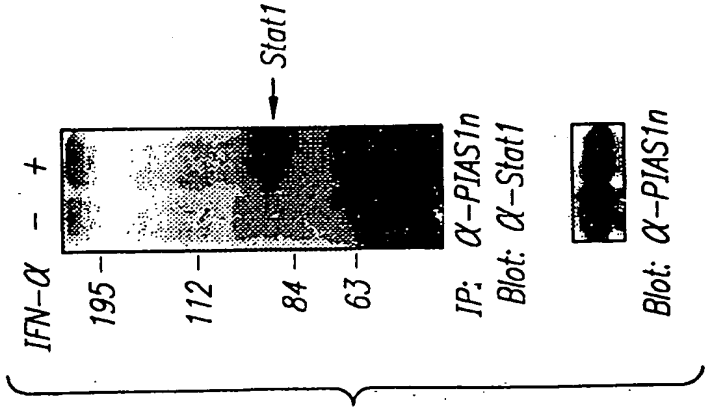

IFN-α - +
195
112
84
63
Stat1
IP: α-PIAS1n
Blot: α-Stat1
Blot: α-PIAS1n

FIG. 8B

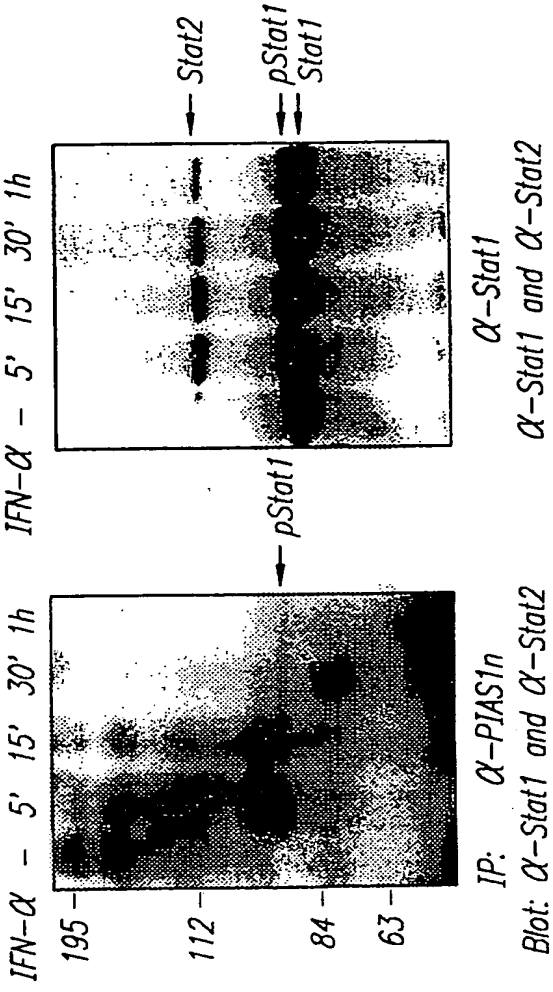

IFN-α - 5' 15' 30' 1h
195
112
84
63
pStat1
IP: α-PIAS1n
Blot: α-Stat1 and α-Stat2
IFN-α - 5' 15' 30' 1h
Stat2
pStat1
Stat1
α-Stat1
α-Stat1 and α-Stat2
PIAS1
Blot: α-PIAS1n

FIG. 8E

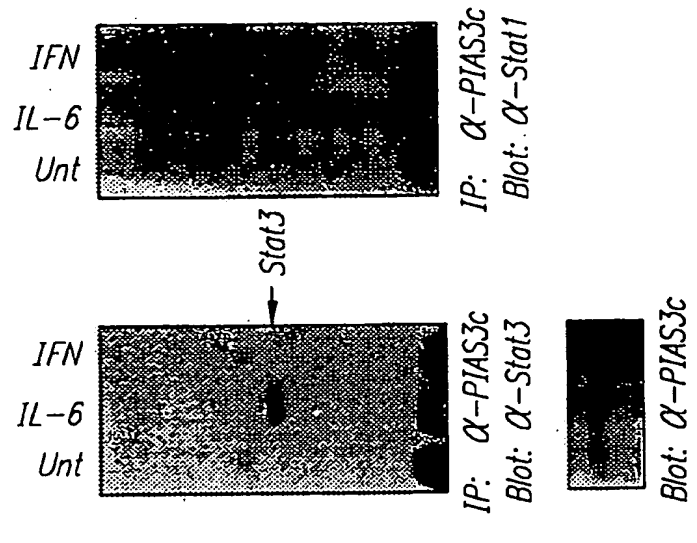


FIG. 8D

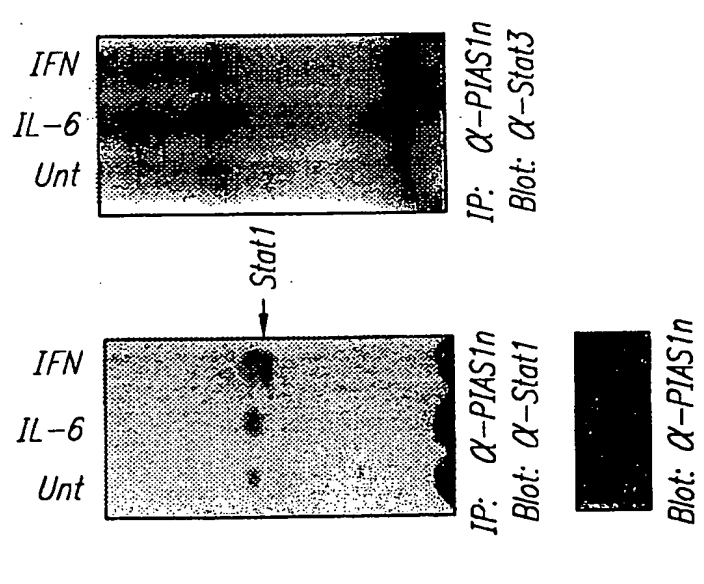
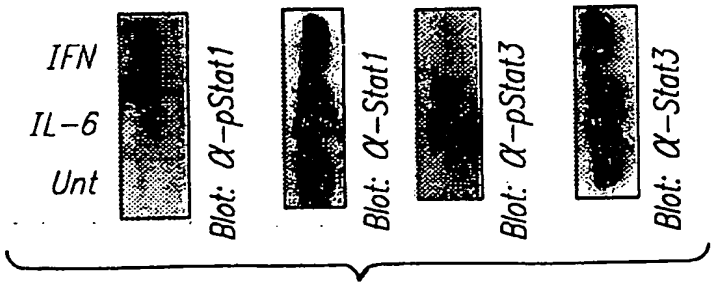


FIG. 8C



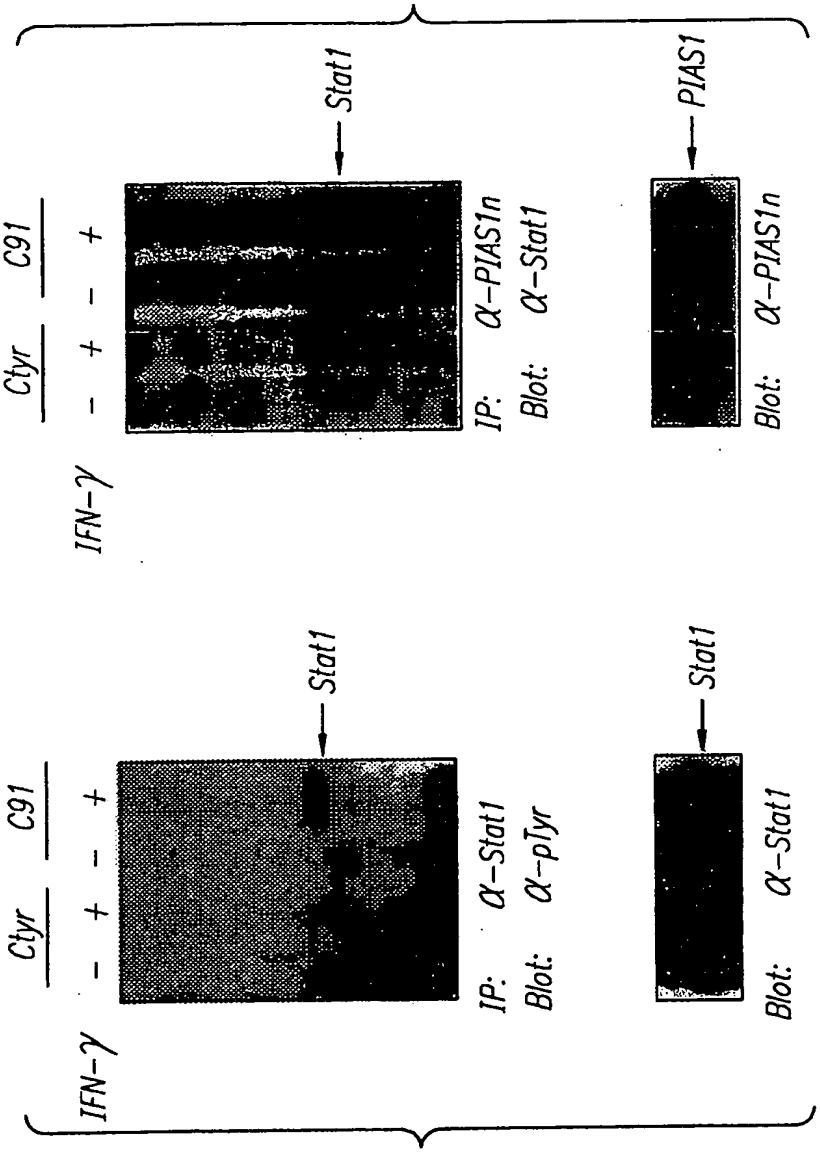

Ctyr C91
IFN-γ - + - +
Stat1
IP: α-Stat1
Blot: α-pTyr
Stat1
Blot: α-Stat1
Ctyr C91
IFN-γ - + - +
Stat1
IP: α-PIAS1n
Blot: α-Stat1
PIAS1
Blot: α-PIAS1n

FIG. 9A

FIG. 9B

FIG. 10-1

```
1/1                                                                 31/11
ATG GCG GAC AGT GCG GAA CTA AAG CAA ATG GTT ATG AGC CTT AGA GTT TCT GAA CTC CAA
M   A   D   S   A   E   L   K   Q   M   V   M   S   L   R   V   S   E   L   Q
61/21                                                               91/31
GTA CTG TTG GGC TAC GCT GGG AGG AAC AAG CAC GGA CGC AAA CAC GAA CTT CTT ACA AAA
V   L   L   G   Y   A   G   R   N   K   H   G   R   K   H   E   L   L   T   K
121/41                                                              151/51
GCC CTG CAT TTG TTA AAG GCT GGC TGT AGT CCT GCT GTA CAA ATG AAA ATT AAA GAA CTC
A   L   H   L   L   K   A   G   C   S   P   A   V   Q   M   K   I   K   E   L
181/61                                                              211/71
TAC AGG AGG CGG TTC CCT CAG AAA ATT ATG ACG CCT GCG GAC TTG TCT ATC CCC AAC GTA
Y   R   R   R   F   P   Q   K   I   M   T   P   A   D   L   S   I   P   N   V
241/81                                                              271/91
CAT TCA AGT CCT ATG CCT CCG ACT CTT TCT CGA TCC ACC ATT CCA CAG CTC ACT TAT GAT
H   S   S   P   M   P   P   T   L   S   P   S   T   I   P   Q   L   T   Y   D
301/101                                                             331/111
GGC CAC CCT GCA TCA TCC CCA CTA CTC CCT GTT TCT CTT CTG GGA CCC AAA CAT GAA CTG
G   H   P   A   S   S   P   L   L   P   V   S   L   L   G   P   K   H   E   L
361/121                                                             391/131
GAA CTC CCA CAT CTC ACG TCA GCG CTG CAC CCA GTC CAC CCG GAC ATA AAG CTG CAG AAG
E   L   P   H   L   T   S   A   L   H   P   V   H   P   D   I   K   L   Q   K
421/141                                                             451/151
CTA CCA TTC TAT GAC CTG TTG GAT GAA CTG ATC AAG CCC ACC AGT CTA GCT TCA GAC AAC
L   P   F   Y   D   L   L   D   E   L   I   K   P   T   S   L   A   S   D   N
481/161                                                             511/171
AGC CAG CGC TTT CGG GAA ACC TGT TTT GCA TTT GCC TTG ACA CCA CAA CAG GTG CAG CAG
S   Q   R   F   R   E   T   C   F   A   F   A   L   T   P   Q   Q   V   Q   Q
541/181                                                             571/191
ATC AGC AGC TCC ATG GAT ATT TCT GGG ACC AAA TGT GAC TTC ACA GTG CAG GTC CAA TTA
I   S   S   S   M   D   I   S   G   T   K   C   D   F   T   V   Q   V   Q   L
601/201                                                             631/211
AGG TTT TGT TTA TCA GAA ACC AGT TGT CCA CAA GAA GAT CAC TTC CCA CCC AAC CTT TGT
R   F   C   L   S   E   T   S   C   P   Q   E   D   H   F   P   P   N   L   C
661/221                                                             691/231
GTA AAA GTG AAT ACA AAA CCT TGC AGC CTT CCA GGT TAC CTT CCA CCT ACT AAA AAC GGT
V   K   V   N   T   K   P   C   S   L   P   G   Y   L   P   P   T   K   N   G
721/241                                                             751/251
GTG GAA CCA AAG CGA CCT AGC CGA CCA ATT AAT ATC ACC TCA CTT GTC CGA TTG TCC ACG
V   E   P   K   R   P   S   R   P   I   N   I   T   S   L   V   R   L   S   T
781/261                                                             811/271
ACA GTA CCA AAT ACC ATT GTT GTT TCT TGG ACT GCA GAA ATT GGA AGA ACC TAT TCC ATG
T   V   P   N   T   I   V   V   S   W   T   A   E   I   G   R   T   Y   S   M
841/281                                                             871/291
GCA GTA TAT CTT GTA AAA CAG TTG TCC TCA ACA GTT CTT CTT CAG AGG TTA CGA GCA AAG
```

FIG. 10-2

```
A  V  Y  L  V  K  Q  L  S  S  T  V  L  L  Q  R  L  R  A  K
901/301                                931/311
GGA ATA AGG AAT CCG GAT CAT TCT AGA GCT TTA ATT AAA GAG AAG TTA ACT GCA GAT TCA
G  I  R  N  P  D  H  S  R  A  L  I  K  E  K  L  T  A  D  S
961/321                                991/331
GAT AGT GAG ATA GCT ACT ACC AGC CTA CGG GTT TCG CTG CTG TGT CCA CTT GGG AAA ATG
D  S  E  I  A  T  T  S  L  R  V  S  L  L  C  P  L  G  K  M
1021/341                               1051/351
CGA CTG ACA ATC CCC TGT CGG GCA CTT ACC TGC TCC CAc ctt cag tgt ttt gat gca act
R  L  T  I  P  C  R  A  L  T  C  S  H  L  Q  C  F  D  A  T
1081/361                               1111/371
ctt tac att caa atg aat gag aaa aaa cca aca tgg gtt tgt cct gtc tgt gat aag aag
L  Y  I  Q  M  N  E  K  K  P  T  W  V  C  P  V  C  D  K  K
1141/381                               1171/391
gcc cca tat gaa cac ctt att att gac ggg ttg ttt atg gaa att cta aag tac tgc aca
A  P  Y  E  M  L  I  I  D  G  L  F  M  E  I  L  K  Y  C  T
1201/401                               1231/411
gac tgt gac gag ata cag ttt aag gag gat ggc tcg tgg gct cca atg agg tca aag aag
D  C  D  E  I  Q  F  K  E  D  G  S  W  A  P  M  R  S  K  K
1261/421                               1291/431
gag gtt caa gaa gtc act gcc tcc tac aat gga gtt gat ggt tgc ttg agc tcc aca ttg
E  V  Q  E  V  T  A  S  Y  N  G  V  D  G  C  L  S  S  T  L
1321/441                               1351/451
gag cat cag gta gcg tcc cac aac cag tcc tca aat aaa aac aag aaa gtc gag gtc att
E  H  Q  V  A  S  H  N  Q  S  S  N  K  N  K  K  V  E  V  I
1381/461                               1411/471
gac cta acc att gac agc tcg tca gat gaa gag gag gaa gAA CCC CCT GCC AAG AGG ACC
D  L  T  I  D  S  S  S  D  E  E  E  E  E  P  P  A  K  R  T
1441/481                               1471/491
TGT CCT TCC CTG TCT CCT ACG TCA CCA CTA AGT AAT AAA GGC ATT TTA AGT CTT CCT CAT
C  P  S  L  S  P  T  S  P  L  S  N  K  G  I  L  S  L  P  H
1501/501                               1531/511
CAA GCC TCG CCT GTG TCC CGC ACC CCA AGC CTT CCT GCT GTA GAT ACA AGC TAC ATC AAC
Q  A  S  P  V  S  R  T  P  S  L  P  A  V  D  T  S  Y  I  N
1561/521                               1591/531
ACC TCC CTC ATC CAG GAC TAC AGG CAC CCC TTC CAC ATG ACG CCT ATG CCT TAT GAC TTA
T  S  L  I  Q  D  Y  R  H  P  F  H  M  T  P  M  P  Y  D  L
```

FIG. 10-3

```
1621/541                                                    1651/551
CAA GGA TTA GAT TTC TTT CCT TTC TTA TCA GGA GAC AAT CAG CAT TAC AAC ACC TCC CTG
Q   G   L   D   F   F   P   F   L   S   G   D   N   Q   H   Y   N   T   S   L
1681/561                                                    1711/571
CTA GCC GCA GCT GCA GCG GCG GTC TCA GAT GAC CAG GAC CTC CTG CAC TCC TCC CGG TTT
L   A   A   A   A   A   A   V   S   D   D   Q   D   L   L   H   S   S   R   F
1741/581                                                    1771/591
TTC CCG TAT ACC TCC TCG CAG ATG TTT CTC GAC CAG CTA AGT GCA GGA GGG AGC ACA TCT
F   P   Y   T   S   S   Q   M   F   L   D   Q   L   S   A   G   G   S   T   S
1801/601                                                    1831/611
CTG CCA GCC ACC AAC GGA AGC AGT AGC GGC AGC AAC AGC AGC CTT GTG TCT TCC AAC AGT
L   P   A   T   N   G   S   S   S   G   S   N   S   S   L   V   S   S   N   S
1861/621                                                    1891/631
CTG AGA GAG AGC CAT GGC CAT GGT GTG GCC AGC AGG AGC AGC GCA GAC ACA GCG TCC ATC
L   R   E   S   H   G   H   G   V   A   S   R   S   S   A   D   T   A   S   I
1921/641                                                    1951/651
TTT GGC ATC ATA CCA GAC ATT ATC TCA TTG GAC TGA
F   G   I   I   P   D   I   I   S   L   D   *
```

FIG. 11-1

```
1/11                                                                31/11
ATG GTG ATG AGT TTC CGA GTG TCT GAG CTC CAG GTG CTC CTC GGC TTC GCT GGC AGG AAC
M   V   M   S   F   R   V   S   E   L   Q   V   L   L   G   F   A   G   R   N
61/21                                                               91/31
AAG AGT GGG CGG AAA CAC GAG CTG CTG GCC AAG GCC CTG CAC CTC CTC AAG TCT AGC TGC
K   S   G   R   K   H   E   L   L   A   K   A   L   H   L   L   K   S   S   C
121/41                                                              151/51
GCC CCC AGC GTC CAG ATG AAG ATC AAA GAA CTT TAT CGC AGG CGC TTT CCC CGG AAG ACC
A   P   S   V   Q   M   K   I   K   E   L   Y   R   R   R   F   P   R   K   T
181/61                                                              211/71
CTG GGG CCT TCT GAT CTC TCC TTG CTT TCT TTG CCC CCT GGC ACC TCT CCT CCT GTG CAC
L   G   P   S   D   L   S   L   L   S   L   P   P   G   T   S   P   P   V   H
241/81                                                              271/91
CCC GAT GTC ACC ATG AAG CCA CTG CCC TTC TAT GAA GTC TAT GGG GAG CTC ATC CGA CCC
P   D   V   T   M   K   P   L   P   F   Y   E   V   Y   G   E   L   I   R   P
301/101                                                             331/111
ACC ACC CTT GCG TCC ACC TCC AGC CAG AGG TTC GAG GAA GCC CAC TTC ACC TTC GCG CTC
T   T   L   A   S   T   S   S   Q   R   F   E   E   A   H   F   T   F   A   L
361/121                                                             391/131
ACT CCC CAG CAG CTG CAG CAG ATT CTC ACG TCC AGG GAA GTT CTG CCA GGA gCC AAG TGT
T   P   Q   Q   L   Q   Q   I   L   T   S   R   E   V   L   P   G   A   K   C
421/141                                                             451/151
GAT TAC ACC ATA CAA GTG CAG CTC AGA TTC TGT CTC TGT GAG ACC AGC TGC CCT CAG GAG
D   Y   T   I   Q   V   Q   L   R   F   C   L   C   E   T   S   C   P   Q   E
481/161                                                             511/171
GAC TAT TTC CCC CCT AAC CTC TTT GTT AAG GTT AAT GGG AAA CTC TGC CCC CTG CCG GGT
D   Y   F   P   P   N   L   F   V   K   V   N   G   K   L   C   P   L   P   G
541/181                                                             571/191
TAC CTC CCT CCA ACC AAG AAT GGA GCT GAG CCC AGA gGC CCA GCC GTC CGA TCA ACA TCA
Y   L   P   P   T   K   N   G   A   E   P   R   G   P   A   V   R   S   T   S
601/201                                                             631/211
CAC CCT TGG CTC GAC TCT CAG CCA CTG TCC CCA ACA CCA TCG Tta TTA ATT GGT CAT CTG
H   P   W   L   D   S   Q   P   L   S   P   T   P   S   L   L   I   G   M   L
561/221                                                             691/231
AGT TTG GAC GGA ATT ACT CCT TGT CCG TGT CTG GTG AGG CAA TTG ACT GCA GGG ACC CTT
S   L   D   G   I   T   P   C   P   C   L   V   R   Q   L   T   A   G   T   L
721/241                                                             751/251
CTA CAA AAA CTC AGA GCC AAG GGG ATC CGG AAT CCA GAC CAT TCC CGG GCA CTG ATC AAG
L   Q   K   L   R   A   K   G   I   R   N   P   D   H   S   R   A   L   I   K
781/261                                                             811/271
GAG AAA CTG ACT GCT GAC CCC GAC AGT GAA GTG GCT ACT ACA AGT CTc CCG GGT GTC ACT
E   K   L   T   A   D   P   D   S   E   V   A   T   T   S   L   P   G   V   T
841/281                                                             871/291
CAT GTG CCC GCT AGG AAG ATG CGC CTG ACT GTC CCG TGT CGT GCC CTC ACC TGT GCC CAT
H   V   P   A   R   K   M   R   L   T   V   P   C   R   A   L   T   C   A   H
```

FIG. 11-2

```
901/301                                                 931/311
CTG CAG AGT TTC GAT GCT GCC CTT TAT CTA CAG ATG AAT GAG AAG AAG CCG ACA TGG ACC
L   Q   S   F   D   A   A   L   Y   L   Q   M   N   E   K   K   P   T   W   T
961/321                                                 991/331
TGT CCT GTG TGT GAC AAG AAG GCT CCC TAT GAa TCG CTG ATT ATT GAT GGT TTA TTC ATG
C   P   V   C   D   K   K   A   P   Y   E   S   L   I   I   D   G   L   F   M
1021/341                                                1051/351
GAA ATT CTT AAT TCC TGT TCG GAT TGT GAT GAg ATC CAG TTC ATG GAA GAT GGA TCC TGG
E   I   L   N   S   C   S   D   C   D   E   I   Q   F   M   E   D   G   S   W
1081/361                                                1111/371
TGT CCG ATG AAA CCC AAG AAG GAG GcA TCA GAG GTT TGC CCC ccg cca ggg tat ggg ctg
C   P   M   K   P   K   K   E   A   S   E   V   C   P   P   P   G   Y   G   L
1141/381                                                1171/391
gat ggt ctc cag tac agc gca gtc cag gag gga att cag cca gag agt aag aag agg gtc
D   G   L   Q   Y   S   A   V   Q   E   G   I   Q   P   E   S   K   K   R   V
1201/401                                                1231/411
gaa gtc att gac ttg acc atc gaa agc tca tca gat gag gag gat ttg ccc ccc acc aag
E   V   I   D   L   T   I   E   S   S   S   D   E   E   D   L   P   P   T   K
1261/421                                                1291/431
aag cag tgc Tct gtc acC tCa gcg gcc att cca gcC ctt ttG gga agc aaa gga gTc ctg
K   Q   C   S   V   T   S   A   A   I   P   A   L   L   G   S   K   G   V   L
1321/441                                                1351/451
acA tCt ggt cac cag cca tct tcg gtg Ctg cgg agC cct gca atg ggc aca Ttg ggc agt
T   S   G   H   Q   P   S   S   V   L   R   S   P   A   M   G   T   L   G   S
1381/461                                                1411/471
gat tTC ctg tCt agt ctc ccg gta cat gag tac cca cct gCc ttc cca Ctg ggg gCt gac
D   F   L   S   S   L   P   V   H   E   Y   P   P   A   F   P   L   G   A   D
1441/481                                                1471/491
atc caa ggt tta gat tTa ttt tCt ttc ctt cag aCt gag agt cag cag tac ggc cct tca
I   Q   G   L   D   L   F   S   F   L   Q   T   E   S   Q   Q   Y   G   P   S
1501/501                                                1531/511
gtt atc atC tcg cta gat gaa cag gac acc ttg gGc cat ttC Ttc cag taC cgg ggg acC
V   I   I   S   L   D   E   Q   D   T   L   G   H   F   F   Q   Y   R   G   T
1561/521                                                1591/531
cct tcc cac ttc ctg ggc cca Ctg gcc ccc aca ctg ggg agc tgt cac ggc agt tcc act
F   S   M   F   L   G   P   L   A   P   T   L   G   S   C   H   G   S   S   T
1621/541                                                1651/551
cca gcg ccc cct cct ggt cGT GTC AGC AGC ATT GTG GCT CCT GGG AGC TCC TTG AGg GAA
P   A   P   P   P   G   R   V   S   S   I   V   A   P   G   S   S   L   R   E
1681/561                                                1711/571
GGG CAT GGA GGA CCc CTG CCT TCA GGT CCc TCT TTG ACT GGC TGT CGG TCA GAC GTC ATT
G   H   G   G   P   L   P   S   G   P   S   L   T   G   C   R   S   D   V   I
1741/581
TCC TTG GAC TGA
S   L   D   *
```